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[illegible] Radiations and Life . . . . . *Arthur C. Giese* 223

[illegible] Reduction Patterns in Ciliate Protozoa . . *C. M. Child* 250

[illegible] between the Reproductive Cycle and the Pituitary Gland [illegible] Snake *Thamnophis radix* . . . . . *Edwin S. Ciesiak* 299

[illegible] in Physiological Zoölogy 330

[illegible] Ray Moulton (ed.), The Chemistry and Physiology of Hormones, [illegible]—Arnold Gesell, in collaboration with Catherine S. [illegible], The Embryology of Behavior, *William F. Windle*.

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ULTRAVIOLET RADIATIONS AND LIFE

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I. EFFECTS OF FAR AND NEAR ULTRAVIOLET RADIATIONS

The results of studies on the effects of ultraviolet light upon the life-activities of cells bear upon a number of fundamental problems which have always interested biologists. The ultraviolet radiations of sunlight have been and are a potent factor in the environment of organisms and may have played a part in the origin and early evolution of life. They have also been used as tools in biological and medical investigations, and to enable us to use them more effectively we should better understand the manner in which they act.

Perhaps the earliest recognition of the effects of radiations is to be found in the mythology of primitive peoples, many of whom deified the sun. Thus the Egyptians had Ammon Ra; the Greeks, Helios (Olcott, 1914). The Greeks also recognized the therapeutic value of sunlight as a general body tonic and practiced sun-bathing in their solaria. Herodotus tells us:

> Exposure to the sun is eminently necessary to those who are in need of building themselves and of putting on weight. . . . . In the winter, spring and fall the rays should be made as much as possible to fall directly on the sick, but during the summer one must temper this method for the sick patients because of the excess of heat. It is principally the back which should be exposed to the sun or to the fire as the nerves obeying the will are located mainly in this region and if these nerves are maintained in a state of mild heat the whole body will benefit by it [Mayer, 1932, p. 36].

The Romans continued this practice, and sun-bathing has survived into modern times.

While general irradiation with sunlight has an ancient history, the use of light for specific remedies is very recent. For example, Fiennius in 1735 treated a cancerous condition of the lips with concentrated sunlight. Faure in 1774 irradiated superficial ulcers in the same way, and Löbel in 1815 tried the effect of sunlight on a diseased condition of the eye (Mayer, 1932; Bovie, 1918*a*). However, it was not until the publication of the bactericidal action of sunlight in the fourth quarter of the past century by Downes and Blunt (1877) that a great many investigations were made, reaching a culmination at the turn of the century. Among the studies inspired by Downes and Blunt were those of Finsen, who in turn stimulated much work on animals and bacteria. Finsen demonstrated that concentrated sunlight or

carbon-arc radiations would cure ulcers of lupus vulgaris, a skin tuberculosis found in Scandinavia (Finsen and Forschhammer, 1904; Lomholt, 1940). For this he received the Nobel Prize. Finsen himself recognized that results with complex animals, such as man, could best be interpreted by experiments performed on simpler organisms; and he encouraged work using protozoans and bacteria, along with the practical studies on man, in the Finsen Medical Institute for the Study of Effects of Light, which had been built for him by the admiring citizens of Copenhagen. Thus, for example, Dreyer (1903, 1904) studied the effects of ultraviolet rays on protozoans; Bie (1905*a*, *b*), on bacteria; and Dreyer and Hanssen (1907), on proteins. These studies and others, as well as considerations of apparatus and methods, are summarized in Gunni Busck's "Lichtbiologie" (1904) and in other publications of Finsen's Institute.

The first quarter of the twentieth century is marked by rather sporadic studies, after which intensive interest in effects of ultraviolet radiations was revived; and an extensive literature has developed. It is now well recognized that all types of animal cells, such as protozoans, ova, and blood cells, are similarly sensitive to ultraviolet radiations, as are small transparent animals and tissue cultures of higher animals; in other words, any protoplasm exposed to the short ultraviolet radiations is injured or destroyed (Duggar, 1936, chap. xviii). When the protoplasm in superficial cells of higher animals is exposed to the ultraviolet, it is destroyed, as manifested by sunburn and snow blindness. The deeper cells of the body are not injured, simply because the radiations, being strongly absorbed by the superficial cells, do not reach them. Compared to animal cells, plant cells are extremely resistant to ultraviolet radiations (Bucholtz, 1931), probably because of the strong absorption of the short radiations by the cuticle and cell walls (Kohler, 1904; Schulze, 1909). Superficial cells are most readily affected, deeper tissues requiring prolonged exposure (Arthur and Newell, 1929; Duggar, 1936, chaps. xxiv and xxvi). Some comparisons are given in Table 1.

It is not the purpose of this paper to summarize the results of studies on multicellular animals or plants (see Laurens, 1933) except incidentally, nor to point out the medical applications that have been made of the ultraviolet rays (see Mayer, 1932, and Ellinger, 1941), since this would be impossible within the confines of one report. Rather, it is our purpose to limit the discussion entirely to the fundamental problem which underlies all of these—namely, the effect of these radiations upon the cell and cell activities.

The center of interest in studies on the effects of ultraviolet radiations upon organisms has varied from time to time as specific phases were solved and as investigators moved on to another aspect of the field of inquiry. Some of the earlier problems must be briefly reviewed here both to clarify the issues and to set the stage for the problems current at the moment. One of the issues which troubled early investigators was: Is the effect of sunlight and of artificial radiations an effect upon the medium or upon the organism? Owing to bad techniques, there was a prolonged controversy; but as early as 1905 Bie conclusively demonstrated that irradiation of organic media can result in the formation of materials toxic to organisms subsequently inoculated but that the dosage required to accumulate such toxic materials is exceedingly large, compared with the dosage required to kill the organisms by direct irradiation. This fact has been demon-

strated again and again by innumerable investigators as a check on their experimental conditions.[1,2]

Another problem which was of much concern for a time is the relative effectiveness of the far and the near regions

TABLE 1

COMPARISON OF DOSAGES REQUIRED TO EVOKE VARIOUS EFFECTS*

Effect	λ in A	Dosage in Ergs/Mm²	Citation
Perceptible polarity induction in *Fucus*	2804	1.2	Whitaker, 1941
90 per cent polarity induction in *Fucus*	2804	120	Whitaker, 1941
Perceptible retardation of cleavage of sea-urchin eggs fertilized by irradiated sperm	2654	10	Giese, 1939*c*
Doubling of time of third-cleavage eggs fertilized by irradiated sperm	2654	200	Giese, 1939*c*
Inhibition of neural-fold closure along one-half the length in 50 per cent of embryos	2576	77–92†	Davis, 1944
	2804	71–95†	
Bactericidal effect, 50 per cent	2654	80–90	Gates, 1929*a*
Just perceptible retardation of cleavage of irradiated sea-urchin eggs	2804	50	Giese, 1938*b*
Doubling of time of third cleavage in the above	2804	1,400	
Doubling of time for excystment of *Colpoda*	2654	750	Giese, 1941*b*
Doubling of time for third division after irradiation in *Paramecium*, fed	2654	1,800	Giese, 1939*b*
Doubling of time for third division after irradiation in *Paramecium*, starved	2654	250–400	Giese, unpublished
80 per cent activation of sea-urchin eggs	2260	518–970	Hollaender, 1938
50 per cent inhibition of yeast budding	2654	450	Oster, 1934
Inhibition of growth of chick mesenchyme in tissue culture	2654	500	Mayer and Schreiber, 1934
Induction of mutations in corn pollen‡	2654	1000–2000	Stadler and Uber, 1942
50 per cent killing of corn-smut sporidia	2654	1,700	Landen, 1939
50 per cent killing of corn-smut chlamydospores	2654	15,000	Landen, 1939
Increase in time of ciliary reversal of paramecia	2804	2800	Giese and Crossman, 1944, unpublished
50 per cent immobilization of paramecia	2804	11,200	Giese and Leighton, 1935*a*
50 per cent vesiculation of paramecia	2804	15,300	Giese and Leighton, 1935*a*
Inhibition of rhizoid formation in *Fucus*	2804	24,000	Whitaker, 1941
Minimal perceptible erythema	2967	2400–5000	Coblentz, Stair, and Hogue, 1932
No perceptible effects on *Mnium* gametophyte after a dosage of	2654	221,800	Bucholtz, 1931§
	2804	123,800	
No perceptible effect on stamen hairs of *Tradescantia*	2654	86,400	Bucholtz, 1931§
	2804	57,600	
No measurable effect on permeability of sea-urchin eggs	2804	3,000	Reed, 1944
	2483	3,000	
No perceptible effect on permeability of erythrocytes	2537	32,000	Giese and Parpart, 1940, unpublished
	2537	64,000	
Inactivation and loss of fertilizing power of sperm of sea urchin	2654	6,000	Giese, 1939*c*

* Many of the dosages listed are approximate, since they had to be estimated from graphs.

† Values corrected for absorption by vitelline membrane.

‡ For mutations in *Trichophyton*, Emmons and Hollaender (1939) find a dosage of 0.01 erg/spore optimal. On the basis of the diameter of the spores (Hollaender and Emmons, 1939), the dosage in ergs/mm² may be estimated as between 1200 and 2400.

§ Irradiation with unfiltered mercury-arc radiation was effective in killing cells of *Mnium* and *Tradescantia*. This indicates that the dosages of monochromatic light were merely insufficient for the purpose.

[1] Bedford (1927) thinks that the toxic material produced by the ultraviolet in the medium might be hydrogen peroxide; Baumgärtner (1936), studying the nature of the changes which render the medium toxic, shows that in the particular instance investigated it is a fall in pH. When the pH is adjusted, the medium is no longer toxic. Pratt (1936), in similar studies, concludes that a fall in pH is not the only change, since media adjusted to the proper pH may still remain toxic, the toxic material being thermostable and nonvolatile.

[2] Forbes and Daland (1923) have shown that ozone which is toxic to organisms is formed by wave lengths of 1850 A and shorter. This does not concern us, since the studies reported here are for wave lengths longer than 2000 A.

of the ultraviolet spectrum. As shown in Figure 1, the ultraviolet spectrum ranges from about 150 A[3] at the short end to 3900 A at the long end of the spectrum. The portion from 150 to 2000 A constitutes the Millikan, Lyman, and Schumann regions, which are absorbed by most materials, including air and water, and are therefore not used in biological work excepting for a few studies in the Schumann region (Bovie, 1918*a;* Forbes and Daland, 1923). The spectrum generally employed is from 2000 to 3900 A. The portion from 2000 to 3000 A is usually called the "far" or "short" ultraviolet, from 3000 to 3900 A the "near"

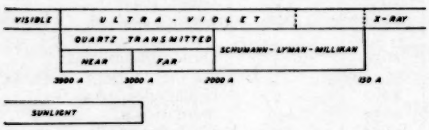

FIG. 1.—Subdivisions of the ultraviolet spectrum

or "long" ultraviolet. Numerous studies, which need not be cited here, have shown that the far ultraviolet is bactericidal and destructive to animals and plants, and therefore it is often called the *abiotic* ultraviolet; whereas the near ultraviolet is relatively innocuous and, according to some, stimulating, and therefore it is sometimes called the *biotic* ultraviolet. Between the two there is a transition zone. The relatively harmless nature of the biotic radiations has been adequately demonstrated with bacteria, paramecia, sea-urchin eggs, protozoan cysts, and roundworm eggs. The data are summarized in Table 2. As the transition zone is approached, some destructive action becomes perceptible if the test process is sufficiently delicate, as, for example, excystment of *Colpoda* or the division rate of sea-urchin eggs. But in these cases much larger doses are required than those used at shorter wave lengths to produce the same effect.

The contrast in action of near and far ultraviolet may appear surprising but becomes rational when we compare these components in the sunlight reaching the surface of the earth. The ultraviolet spectrum of the sun covers the span from 2890 to 3900 A. The intensity of the ultraviolet radiations varies with time of day, being greatest at midday; with clarity of the atmosphere, being greatest when the atmosphere is clear; with altitude, being more intense at higher altitudes other conditions being equal; with latitude, increasing toward the Equator; with seasons of the year, being greatest in summer in the Northern Hemisphere; and with sunspot activity, increasing with sunspots (see Laurens, 1933). But in no case—not even from the heights of a mountain or aloft in a balloon—is the radiation shorter than 2890 A (Fabry and Buisson, 1930).[4] Furthermore, the intensity of the radiations in the far ultraviolet from 2890 to 3000 A is extremely low, compared to the intensity of the near ultraviolet. Considering the general adaptiveness of organisms to their environment, we might expect the near ultraviolet, which is relatively intense in sunlight, to be harmless; while the far ultraviolet, which is comparatively weak and to which organisms are exposed only for brief periods of time, might still be destructive.

II. ULTRAVIOLET ACTION SPECTRA

One of the problems which occupies the center of interest at the present time is the relative effectiveness of various wave lengths in the far ultraviolet. In a study of the effects of ultraviolet radiations upon cells the main variables are intensity, wave length, and dosage. It has been shown repeatedly that the reci-

[3] "A" stands for the angstrom unit, 10^{-8} cm.; 10 A = 1 mμ; 1000 mμ = 1 μ.

[4] Götz (1931) cites one record of 2863 A.

procity law of Bunsen and Roscoe holds over wide limits—that is, provided the product of the intensity times the time is constant the effects are the same. The widest range in intensities are those recently reported by Rentschler, Nagy, and Mouromseff (1941), in which a several-million-fold variation produced similar effects. When the intensity becomes very low and the time very long, so that the organisms undergo development during the period of exposure, the results may not be the same, owing to the presence of more sensitive stages in the cycle of development. Provided precaution is taken against such errors, the reciprocity law is found to hold. In the following discussion the intensity will generally be ignored, since it varied only over a relatively small range in any given experiment quoted.

The effectiveness of different wave lengths can be compared by the dosages required to produce a given effect. This gives the so-called *action spectrum*. Warburg (1927) used this concept successfully in his classical identification of the *Atmungsferment* by the release of the carbon monoxide inhibition with visible monochromatic light of different wave lengths. Among the first to apply this concept to the action of ultraviolet light upon cells was Gates (1930), who found the bactericidal action spectrum for

TABLE 2

THE LONG-WAVE-LENGTH LIMIT OF ACTION IN THE ULTRAVIOLET

λ in A	Effect	Dosage in Ergs/Mm²	Citation
3025.....	Bactericidal effect (*Staphylococcus aureus*)	3,150	Gates, 1929*a*
	Lethal action of corn-smut sporidia (*Ustilago zeae*)	100,000	Landen, 1939
	Doubling of time of excystment of *Colpoda*	5,000	Giese, 1941*b*
	Doubling of time of third cleavage of sea-urchin egg	4,000	Giese, 1938*a*
	Lethal action on paramecia	28,000	Giese and Leighton, 1935*a*
3130.....	Bactericidal effect, 50 per cent	25,000	Duggar (1936, p. 1125)
	Retardation of cleavage of sea-urchin eggs fertilized by irradiated sperm	4,000	Giese, 1939*c*
	Slight retardation of excystment of *Colpoda*	30,000	Giese, 1941*b*
	Slight retardation of cleavage of sea-urchin eggs	12,600	Giese, 1938*b*
	Slight retardation of division of paramecia;	40,000	Giese, unpublished
	Definite retardation	80,000	
	No visible signs of injury after*	200,000	
	Small number of endosperm-deficiency mutations in corn	512,000	Stadler and Uber, 1942
	No lethal action on sporidia of corn smut, *U. zeae*, even after a dosage of	1,500,000	Landen, 1939
3660.....	Retardation of cleavage of sea-urchin eggs fertilized by irradiated sperm	16,000	Giese, 1939*c*
	Very slight retardation of cleavage of irradiated sea-urchin eggs	74,500	Giese, 1938*b*
	No perceptible effect on excystment time of *Colpoda*	84,000	Giese, 1941*b*
3500–4900...	Bactericidal effect on *Escherichia coli*	2,000,000	Hollaender, 1943
	Lethal effect on nematode eggs	12,300,000	Jones and Hollaender, 1944

* Weinstein (1930), working with paramecia, gives 19,621 ergs/mm² as lethal at 3130 A; Wyckoff (1932), using the same wave length on colon bacteria, gives 5,200 ergs/mm² as 50 per cent bactericidal. The purity of the light at this wave length is not given; it is possible that sufficient scattered radiations of shorter wave length account for the lethal action with such small dosages.

Bacillus coli given in Figure 2. Sonne (1929) made a similar study not only for the bactericidal effect but for other light-effects as well; but since the data published by Gates are much more complete, they will be used in this discussion. Various types of action spectra are summarized in Table 3.

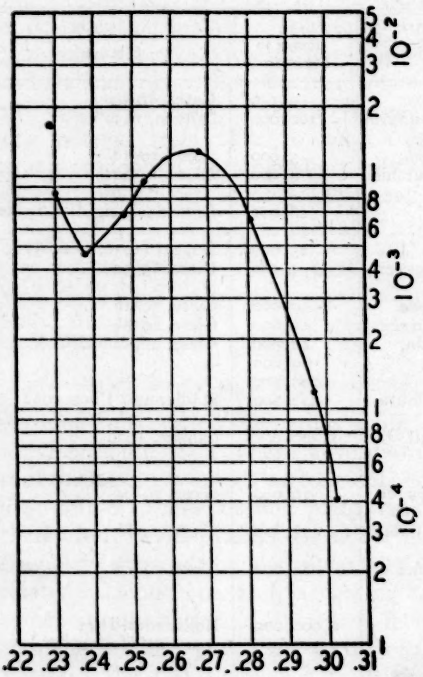

FIG. 2.—Action spectrum for the bactericidal effect (Gates, 1930, p. 35); abscissa, λ in μ; ordinate, relative effectiveness in reciprocals of the dosage required.

Since the action spectrum is so important in the analysis of any photobiological reaction, it seems desirable to give as an example a brief description of the techniques used in the determination of the bactericidal action spectrum. The bacteria are poured or sprayed onto nutrient agar plates, great care being taken to get an even and thin layer over the plates. A series of strips on such a plate is exposed to graded dosages of ultraviolet alternately with control strips. After a given period of incubation the number of colonies on the strips is counted, the values so determined are plotted as in Figure 3, and a smooth curve is drawn through the average values. The same experiment is carried out with each wave length tested in turn. The smoothed curves for all wave lengths are plotted in Figure 4; and the amount of energy required to kill 50 per cent of the organisms at each wave length is determined from the curve, and these values are plotted as in Figure 2, giving the action spectrum. All the action spectra spoken of in this paper are of this sort, although each organism is handled according to its need and the exact criteria of effective action vary with the organisms or processes studied.

Use of the sun as a source of radiations was early abandoned (Arloing, 1887) because investigators were at the mercy of the caprices of the weather and because adequate analysis of the action of sunlight itself is impossible with the use of the limited ultraviolet spectrum available in sunlight, since the action-spectrum method of analysis requires work with short wave lengths. Consequently, the bulk of the work has been done with various artificial sources of ultraviolet radiations, such as carbon arcs, quartz mercury-vapor lamps, and spark-discharge and vapor-discharge tubes.

It goes without saying that such determinations are impossible without monochromatic light of known intensity (Forbes, 1928). Generally monochromators of natural quartz are used. The desired wave length is picked out from the widely spaced lines and focused on the object with a quartz lens. The intensity is most frequently determined with a thermopile having a nonselective black surface coating, such as zinc black, which

absorbs all wave lengths equally. The thermopile is calibrated against blackbody radiators (see Forsythe, 1937). The intensity is thus determined in absolute units, and experiments performed by another at about 2600 A; a minimum in the region of 2500 A; and a falling-off toward zero in the region of 3050 A (Fig. 2).

This type of action spectrum is found

TABLE 3

SUMMARY OF ACTION SPECTRA

Absorption Maxima	Absorption Minima	Possible Absorbing Substance	Effects	Citations
1. Below 2400 and at 2600 A	About 2500 A	Nucleoprotein	Bactericidal and fungicidal	Gates, 1930; Landen, 1939
			Mutations	Stadler and Uber, 1942
			Inhibition of growth of tissue cultures	Mayer and Schreiber, 1934
			Inhibition of division of starved paramecia; recovery from sublethal doses	Giese, 1939, unpublished
2. Below 2500 and at 2800 A	Between 2500 and 2600 A	Cytoplasmic-type proteins with aromatic amino acids	Retardation of excystment	Giese, 1941*b*
			Prolonging reversal of ciliary activity	Giese and Crossman, unpublished
			Immobilization and vesiculation of paramecia	Giese and Leighton, 1935*a*
			Prevention of hatching of nematode eggs	Hollaender, Jones, and Jacobs, 1940
3. Below 2654 A and increasing with decrease in wave length	.....	Cytoplasmic-type proteins without aromatic amino acids	Activation	Hollaender, 1938
4. As in 3, but below 2800 A	.....	As in 3; or lipids?	Hemolysis	Sonne, 1929
5. 2970 A and 2500 A and below	2800 A	Possibly as in 2	Erythema (and delayed tanning?)	Coblentz and Stair, 1934
6. 3400–3500 A	.....	Precursor of oxidized melanin	Direct or immediate tanning	Henschke and Schulze, 1939*a*; Henschke, 1940
7. About 2570 and 2800 A	Below 2500 A and between 2600 and 2700 A	Vitamin D precursors	Inhibition of neural-fold development	Davis, 1944

different investigators can be directly compared.

The bactericidal action spectrum of Gates has been corroborated by studies on other species of bacteria (see Duggar, 1936, chap. xxxvi). Not all the data are identical, but the variations from the values for different wave lengths found by Gates are relatively minor. In all cases a maximum is found below 2400 A; not only for bactericidal effects but also for inhibition of division of yeast (Oster, 1934, 1935), inhibition of growth of the smut fungus (Landen, 1939), for induction of mutation in the fungus *Trichophyton* (Emmons and Hollaender, 1939), in the fungus Neurospora (Hollaender, Sansome, Zimmer, and Demerec, 1945), and in pollen grains of maize (Stadler and Uber, 1942), for reduction in growth

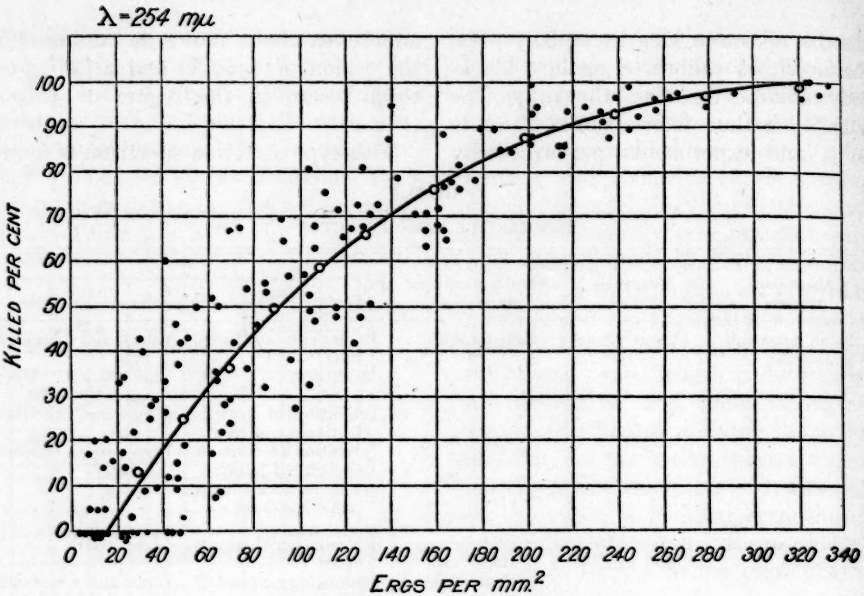

FIG. 3.—Bactericidal curve for one wave length (Gates, 1929*a*, p. 239)

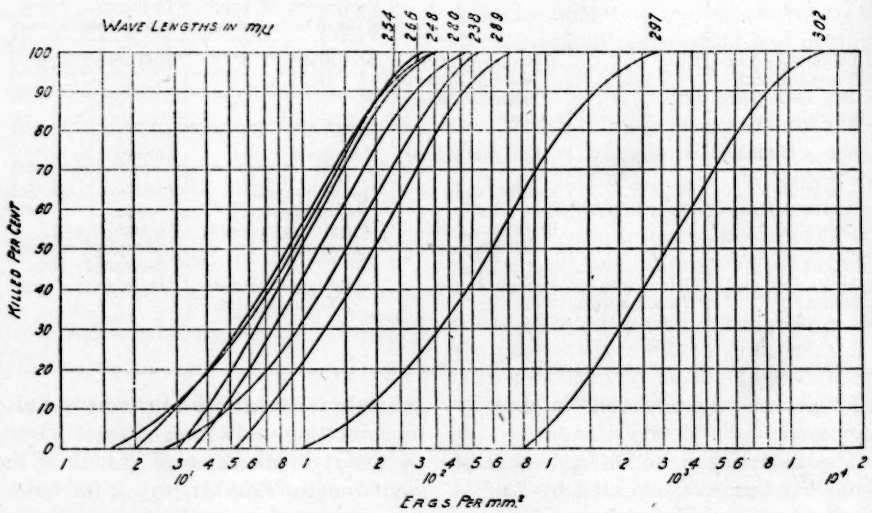

FIG. 4.—Smoothed bactericidal curves for many wave lengths (Gates, 1929*a*, p. 240)

in area of explants of chick-embryo mesenchyme (Mayer and Schreiber, 1934), and for retardation of division of starved paramecia and recovery of paramecia from sublethal dosages (Giese, unpublished). Some of the data are given in Figures 5 and 11.

A second type of action spectrum shows a maximum at about 2800 A, a minimum between 2600 and 2500 A, and a second maximum below 2500 A (Fig. 6). Such an action spectrum is found for retardation of excystment of *Colpoda* (Giese, 1941, and Giese, unpublished), for prolonging the time of reversal[5] of ciliary activity (Giese and Crossman, 1943, unpublished), for immobilization and vesiculation of paramecia (Giese and Leighton, 1935*a* and unpublished data), and for prevention of hatching of nematode eggs (Hollaender, Jones, and Jacobs, 1940). The data are given in Figure 6. The effects on motility are probably

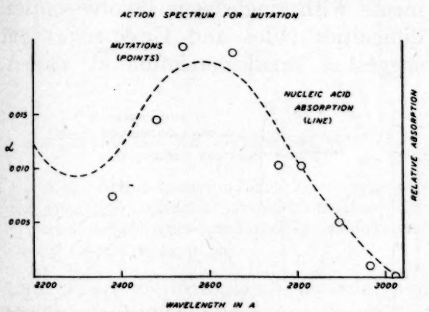

FIG. 5.—Action spectrum for rate of mutation in corn. The value of a, to a first approximation, "should be proportional to the absorption coefficient of the substance responsible for the production of the deficiencies" (mutations) (Stadler and Uber, 1942, p. 110).

measures of a common effect on the motor organelles produced by different degrees of irradiation. Thus the increased time of ciliary reversal is produced by a relatively small dosage, compared to the dosage which results in immobilization and finally in disruption and vesiculation of the protoplasm. The

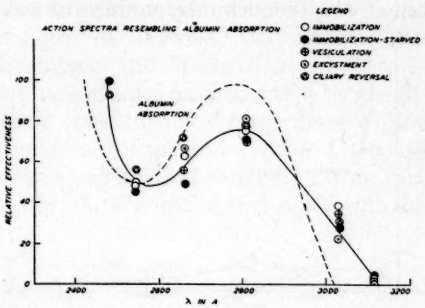

FIG. 6.—Action spectra for immobilization, vesiculation of paramecia (Giese and Leighton, 1935*a*, and Giese, unpublished), increased time of ciliary reversal in paramecia (Giese and Crossman, unpublished), retardation of excystment in *Colpoda* (Giese, 1941*a*, and unpublished).

retardation of excystment is produced by even smaller dosages than the increased time of ciliary reversal (Table 1). It is thus evident that the action spectrum may be the same for effects requiring either very small or very large dosages.

The third and fourth types of action spectra—for activation of unfertilized sea-urchin eggs and for hemolysis—are given in Figure 7. Hollaender (1938) found that fertilization membranes were raised only after irradiation with wave lengths shorter than 2654 A. Sonne's data (1929) for hemolysis are very similar but shifted about 140 A toward the long-wave-length end of the spectrum.

The fifth and sixth types of action spectra in Table 3 are those for erythema and tanning. Erythema is the reddening of the skin which occurs after exposure to various irritants, among them ultraviolet light. In part, owing to different criteria of relative effectiveness being used, the various sets of data are not in com-

[5] Ciliary reversal occurs when normal paramecia are placed in certain solutions to which they have not become adjusted, as, for example, M/50 KCl (Oliphant, 1938). Such reversal will continue for a longer time if the animal is first injured.

plete agreement; but the differences are of secondary order. Coblentz and Stair (1934) have revised these values, and their data are used in Figure 8. Excessive exposure of the skin is followed not only by erythema but by sunburn as well.

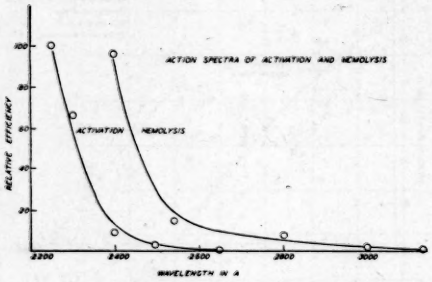

FIG. 7.—Action spectra for hemolysis (Sonne, 1929) and activation of *Arbacia* eggs (Hollaender, 1938).

Erythema is followed by tanning, at least in individuals showing some pigmentation, the red color of the erythema changing almost imperceptibly to tan in the course of a few days. The action spectrum may be similar to that for erythema (Blum. 1941). Tanning of another kind, which follows immediately after irradiation, may occur even without erythema and has an action spectrum different from that for erythema, with a maximum at about 3400–3500 A, as shown in Figure 8 (Henschke and Schulze, 1939*a*, *b;* Henschke, 1940). A seventh type of action spectrum, observed for the inhibition of neural-fold formation in the chick, is shown in Figure 9 (Davis, 1944). This resembles the absorption spectra of vitamin D precursors.

The action spectra summarize the relative efficiency of the different wave lengths in producing the various effects. Provided the incident energy is the same, the wave length which is most efficient in producing a given effect must be most completely absorbed by some component of the cell, since only light which is absorbed can produce activated molecules which lead to photochemical reactions (Grotthus-Draper law). Measurements of absorption of ultraviolet radiations by cells and organisms are difficult to make, owing to the reflection and scattering of these rays from the surface of cells and the uncertainty of corrections for these losses. Gates (1930), using thin layers of bacteria with a small scattering error, records a maximum absorption at 2500–2600 A and, after a small minimum, a second maximum in the short-wave end below 2400 A. Landen (1939), using similar methods, found a similar absorption for the sporidia of a fungus. Studies on paramecia and sea-urchin eggs (Giese and Leighton, 1935*a;* Giese, 1938*a*) indicate maxima at 2804 and 2537 A for the wave lengths tested; but since all large cells scatter much light, the data are not very accurate. Similar experiments with single eggs involve optical difficulties (Vles and Gex, 1934) but suggest a small maximum at 2800 A.

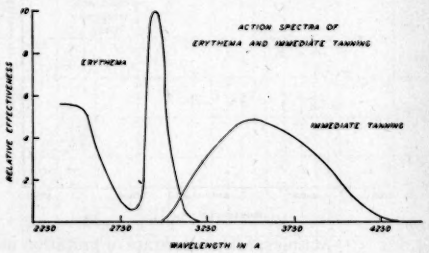

FIG. 8.—Action spectra for erythema (Coblentz and Stair, 1934) and direct or immediate tanning (Henschke, 1940). The incident energy necessary for the latter effect is much greater than for the former, only relative, not absolute, difference being shown in the figure.

Human epidermis also shows a maximum at 2700–2800 A and a minimum at 2500–2600 A (Lucas, 1931). None of the absorption spectra completely correspond to any of the action spectra, the fungicidal and the bactericidal effects coming

closest to matching. These results indicate that injury results not from gross and general absorption by the entire cell but rather from absorption by some particular substance, the absorption spectrum of which corresponds to the action spectrum. One would hardly expect so simple a situation as complete correlation between gross absorption and photobiological effects to hold for so complex

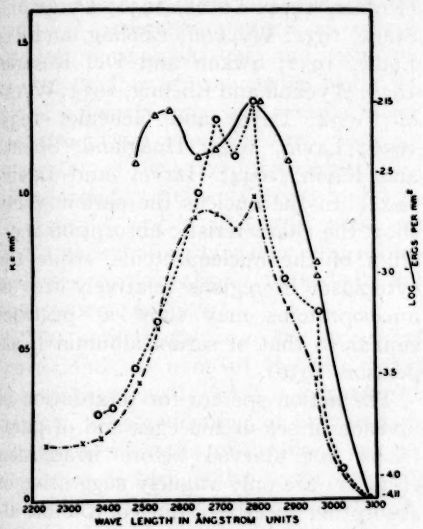

FIG. 9.—Action spectrum for inhibition of neural-tube formation (triangles) compared to absorption by lumisterol (crosses) and 7-dehydrocholesterol (circles) (Davis, 1944, p. 93).

a substance as protoplasm, especially in large animal cells with their many differentiated structures. Therefore, we must look in protoplasm for substances whose absorption spectra correspond to the action spectra observed for animal cells; and not for one substance but several, since seven types of action spectra have been found up to date.

The main constituents of protoplasm are water, proteins, lipids, carbohydrates, salts, and miscellaneous chemicals, such as vitamins, etc. Water absorbs only the very short ultraviolet of the Schumann region, which has seldom been used in biological investigation, but transmits almost completely the spectrum under discussion (Dawson and Hurlburt, 1934). Salts in the dilutions and of the kinds found in protoplasm absorb little in the regions of interest (Crymble, 1912; Brannigan and Macbeth, 1916). A summary of studies on absorption by the organic constituents of protoplasm will be found in Caspersson (1936). Carbohydrates and lipids absorb only rather generally, the absorption increasing with de-

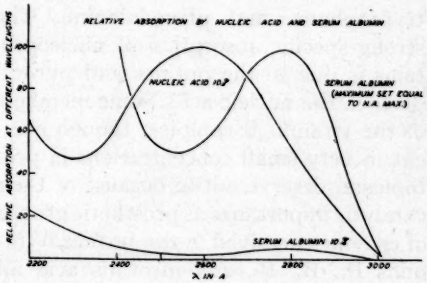
RELATIVE ABSORPTION BY NUCLEIC ACID AND SERUM ALBUMIN
NUCLEIC ACID 10β
SERUM ALBUMIN (MAXIMUM SET EQUAL TO N.A. MAX.)
SERUM ALBUMIN 10β
RELATIVE ABSORPTION AT DIFFERENT WAVELENGTHS
λ IN A

FIG. 10.—Absorption by nucleic acid (Caspersson, 1936) and serum albumin compared (Smith, 1929). For comparison the maxima at 2600 A (for nucleic acid) and at 2800 A (for serum albumin) have been set equal to each other in the upper curves.

crease in wave length. The proteins are divided into two groups with regard to absorption—nucleoproteins and the simple proteins such as albumin, globulin, etc. The latter have an absorption spectrum with a maximum below 2400 A, a minimum at about 2550 A, and a second maximum at 2800 A, as shown in Figure 10. Nucleoproteins, on the other hand, have an absorption spectrum almost like that of the nucleic acid shown in Figure 10. There is a maximum below 2200 A, a minimum at about 2300 A, and a second maximum at about 2600 A. In both types of proteins the absorption falls off to nearly zero at about 3000 A. Compared on an equimolar basis, the nucleo-

proteins absorb ultraviolet radiations much more completely than such proteins as albumin and globulin, typical of the cytoplasm (see Caspersson, 1936). It is important to emphasize that not all nucleoproteins are restricted to the nucleus: chromonucleic acid containing desoxyribose is present in the chromatin of the nucleus, whereas plasmonucleic acid containing ribose is present in the cytoplasm (Mirsky and Pollister, 1942). The strong specific absorption in proteins such as albumin, etc., is due to the aromatic amino acids, such as tyrosine, tryptophane, and phenylalanine. The strong specific absorption of nucleoproteins is due to the purines and pyrimidines in the nucleic acid. Some members of the vitamin B complex, though present in very small concentrations in protoplasm, deserve notice because of their catalytic importance as prosthetic groups of enzymes involved in respiration. Vitamins B_1, B_2, B_6, and nicotinic acid all have strong absorption maxima in the region below 3000 A (see Rosenberg, 1942). In summary, it may be said that, while most compounds absorb ultraviolet light to some extent, the nucleoproteins are the most prominent in this respect.

Let us now see if we can correlate the main types of action spectra with the absorption spectra for certain of the protoplasmic constituents just described. A fairly good correlation is observed between the absorption by nucleoproteins and the action spectra for mutations (Fig. 5), retardation of growth of tissue cultures, retardation of division of paramecia starved before irradiation (Fig. 11), recovery of paramecia from sublethal dosages of radiations (Fig. 11), and the growth-inhibiting effect of ultraviolet upon bacteria and fungi (Figs. 2 and 5). Such a correlation suggests that the absorption by the nucleoproteins mediates these effects, even though, without doubt, radiations are also absorbed by other constituents of the cells. The correlation with nucleoprotein absorption is not surprising, in view of the powerful absorption of short wave lengths shown by the nucleus of the cell, as compared to its cytoplasm. This difference is illustrated graphically in photographs taken with ultraviolet light (Kohler, 1904; Lucas, 1930; Lucas and Stark, 1931; Wyckoff, Ebeling, and Ter Louw, 1932; Swann and Del Rosario, 1932; Wyckoff and Ebeling, 1933; Wyckoff, 1934; Luyet and Gehenio, 1935, 1936; Lavin, 1943; Hoagland, Shank, and Lavin, 1944; Harvey and Lavin, 1944). In the nucleus the chromosomes show the characteristic absorption spectrum of the nucleoproteins, while the cytoplasm in regions relatively free of nucleoproteins may show a spectrum similar to that of serum albumin (Caspersson, 1936).

The action spectra for retardation of division of sea-urchin eggs and of paramecia not starved before irradiation (Fig. 11) are only vaguely suggestive of absorption spectra of nucleoproteins, although this is true of starved paramecia. This paradoxical situation is clarified by a study of the delayed effect of the radiations upon division rather than their immediate retarding effect. If the time required for complete recovery of the normal division rate is plotted against the wave length, the action spectrum obtained is similar to the nucleoprotein absorption-curve. The conclusion is tentatively drawn that the immediate delay is due both to nucleoprotein and to cytoplasmic-protein destruction, since a considerable layer of cytoplasm is interposed between nucleus and the radiations. Recovery from the nucleoprotein injury is slow, leading to a different action spectrum when time for complete

recovery is studied. In starved animals, on the other hand, the nucleoproteins may perhaps be much more readily reached through the more transparent and thinner cytoplasm; consequently, a nucleoprotein type of action spectrum is obtained. Perhaps the same would be found for the sea-urchin egg, but so far it has not been possible to devise a suitable experimental test.

The action spectra for the second group of phenomena—such as ciliary reversal, immobilization, and vesiculation of paramecia; prevention of hatching of pinworm eggs; and retardation of excystment of *Colpoda*—are strongly suggestive of the curve for absorption by cytoplasmic proteins (Figs. 6 and 10). The results are in line with what we might expect if cytoplasmic injuries interfering with various immediate responses were involved.

The action spectrum for erythema corresponds to none of the absorption spectra mentioned; but it is, perhaps, an artifact. The reddening of erythema is due to the dilatation of the blood vessels; the latter, in turn, is due to the action of chemicals released from the tissue injured by the rays. The human skin is a complex organ, made up of epidermis and derma; and the epidermis itself is made up of many thicknesses of cells, forming definite layers. Histological studies have shown that, following irradiation, the breakdown occurs mainly in the prickle-cell layer, many of the cells above this being already dead and cornified or in the process of becoming so. The light which affects the prickle cells must first pass through the cornified cells, and so the erythemal effect is largely a measure of the transparency of these tissues to the erythema spectrum; thus, where the light is transmitted least, as at 2800 A, we find a minimum of erythemal effectiveness (Hauser, 1928). Presumably, the skin cells are no less sensitive than any other cells to the shorter wave lengths of ultraviolet light. We might tentatively suggest that, if the true action spectrum of erythema could be determined, it would prove to be very much like that for the photolethal effects on paramecia (vesiculation and immobilization), but experimental evidence is necessary.[6] However, since the Feulgen staining reaction is much diminished or absent from nuclei of cells of the epidermis given a "sunburn" dosage, the possibility of correlation with nucleoprotein absorption is suggested (Hamperl, Henschke, and Schulze, 1939).

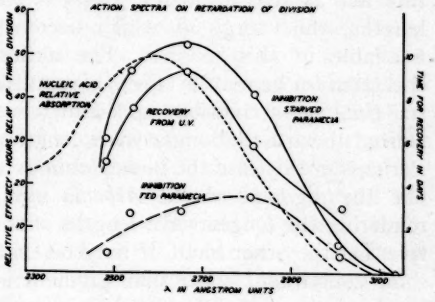

FIG. 11.—Action spectra for retardation of division of paramecia and of recovery of paramecia from sublethal doses of ultraviolet radiations (Giese, unpublished).

The action spectrum in Figure 7 for activation of sea-urchin eggs resembles the short-wave-length end of the action spectra for inactivation of urease and for virus destruction (Hollaender, 1938). The lack of action at 2804 A suggests that the membrane proteins lack the aromatic amino acids; but Hollaender warns against conclusions from the data, since so many organic compounds absorb

[6] It is interesting to point out that accommodation to sunlight after repeated exposure is probably due mainly to the thickening of the corneum of the skin, not to pigmentation, although the latter may play a small role. The deep location of the pigment precludes effective protection of the prickle-cell layer (Guillaume, 1927; Miescher, 1930).

in the short-wave-length end of the spectrum. Activation of the egg presumably occurs entirely as a result of action of light on the cytoplasm, since nonnucleate fragments of the centrifuged eggs will undergo parthenogenetic merogony (Harvey and Hollaender, 1938). Harvey and Hollaender also found that the red halves of centrifuged eggs were activated by longer wave lengths—in fact, up to 3300 A. In such eggs the pigment granules are more concentrated than in the normal egg and may therefore act as sensitizers for longer wave lengths, the energy of which becomes available in this manner. The action spectrum for hemolysis (Fig. 7) is similar to the egg-activation spectrum but shifted toward the longer wave lengths. Perhaps in this case the hemoglobin acts like the pigment of the *Arbacia* eggs, rendering the longer wave lengths effective. On the other hand, it may be that some constituent other than pigment is involved as well. Thus Sonne (1929) suggests lipids as suitable agents, since lipids absorb generally in the ultraviolet, the absorption increasing with decrease in wave length.

The pigmentation which follows immediately after irradiation shows an action spectrum which, after correction is made for the 80 μ of skin through which the light must pass to reach the cells in which the pigment is laid down, suggests that of melanin. It is thought that this substance is photochemically oxidized or that the reducing substances present are destroyed or in some way inactivated by the ultraviolet light (Henschke, 1940).

The last action spectrum which we have considered—the prevention of neural-fold formation—to some extent resembles the curve for absorption by provitamin D compounds (Fig. 9). Since the radiations are filtered by several layers of cells before they can act on the substance in question, a close match cannot be expected.

The importance of vitamin D to the vertebrates is so well known as to need no emphasis here. Since the problem of vitamin D formation is now considered largely chemical rather than biological, only the briefest mention need be made; but the relationship between sunlight ultraviolet and vitamin formation should be pointed out.

While many substances show vitamin D activity, there are two main forms of the vitamin derived from the ergosterol present in plant sterols and from the 7-dehydrocholesterol present in animal sterols (see chap. ix in Duggar, 1936, Vol. 1). Both of these precursors have similar absorption spectra, and the absorption by 7-dehydrocholesterol is shown in Figure 9. Activation occurs throughout this portion of the ultraviolet spectrum in proportion to absorption, the greatest activity being in regions of the spectrum not present in the sunlight reaching the surface of the earth. Intramolecular shifts in bonds occur in the molecule, resulting in the configuration having vitamin D activity (see Rosenberg, 1942). Ergosterol gives rise to calciferol, or vitamin D_2; and 7-dehydrocholesterol gives rise to 7-dehydrocalciferol, or vitamin D_3. Excessive irradiation results in the destruction of vitamin D activity and the formation of a toxic substance, toxisterol. Before the cause of toxicity was discovered, medical men condemned irradiated oils and recommended only the naturally occurring vitamin D in fish oils for therapeutic purposes; but the photochemical changes are now well known and can be controlled, and the vitamin can be obtained in crystalline form if desired.

Activation of provitamin D by sunlight occurs at or near the surface of the skin or on the surface of the hair or

feathers in preening animals. As has already been pointed out in Section I, sunlight is very limited in content of radiations shorter than 3100 A; consequently, the antirachitic radiations are very limited. The intensity of the actinic ultraviolet radiations which pass the ozone umbrella is further attenuated by smoke and fog, both of which scatter the radiations; the former, being opaque, absorbs a considerably greater amount than the latter. This decrease depends upon the concentration of smoke particles and the depth of the layer interposed between the sun and the surface of the earth, being least at noontime, when the sunlight is passing through the shallowest layer of air. The loss in intensity of the short ultraviolet radiations, due to smoke on sunny days in January, may be as much as 42 per cent at 8:00 A.M. in a city such as New York (lower Manhattan) but only 18 per cent at noon, while in June it is about 33 per cent at 10:00 A.M. and 6 per cent at noon. The loss is greater on foggy days. Even in places of low humidity, dust definitely decreases the penetration of ultraviolet light; thus, in the Red Sea region and in Arabia the intensity of the short ultraviolet is very low, and in the central valley of California the haze which is usually present through the summer so reduces the intensity of the antirachitic radiations that a mild form of rickets is found (Laurens, 1933). This seems a paradox, considering the sunny summer; but it may be due largely to the fact that exposure to the sun is avoided during the midday period, when the ultraviolet is most intense, owing to the heat at this time. Fogs and clouds are less effective in reducing the ultraviolet radiations, as attested by sunburns received at the seacoast even on hazy or foggy days. Because of the close relationship between the radiations causing sunburn and those preventing rickets, the erythemic or sunburn activity of the sun is often taken as an index of its antirachitic power.

As a result of fog and smoke, the short-wave-length limit of ultraviolet radiations in New Orleans is seldom less than 3000 A; in fact, on only one June day in four years did the spectrogram extend to 2990 A, and in winter it seldom extends to below 3060 A except on a clear day after a storm. Air pollution, clouds, and haze may reduce the limit to 3100 A even in August. Where less fog and smoke prevail and where the incident radiations are more intense, somewhat shorter limits may be found (Laurens, 1933, chap. i). In any case, the intensity of the radiations shorter than 3130 A constitute, at best, only about 0.1 per cent, and all the ultraviolet radiations, only from 1 to 5 per cent, of the radiations of the sun (Duggar, 1936, Vol. 1, chap. iv). It is thus apparent why fortification of the diet of children with vitamin D is necessary in certain localities, especially cities.

Since substances with provitamin D properties are found in earthworms, snails, and periwinkles, etc., it is possible that compounds similar to vitamin D may have a role in invertebrates as well as vertebrates. Vitamin D may be active not only in the metabolism of the bone, as indicated by its action in rickets, but in general growth metabolism as well. Vitamin D deficiency interferes with phosphorylations, suggesting a role in carbohydrate and fat metabolism. The biochemistry of vitamin D activity thus promises an interesting future (Rosenberg, 1942).

One other topic deserving passing notice is the effect of ultraviolet radiations upon other vitamins. This effect is presumably destructive, since absorption of ultraviolet radiations by solutions of vitamin A, several members of the B

complex, and vitamin C occurs between 2000 and 3000 A, falling off rapidly toward 3000 A (Morton, 1942; Uber and Verbrugge, 1940*a*, *b*). Vitamin B_2 is light-sensitive to considerably longer wave lengths. No action spectrum for a photobiological effect yet presented corresponds with destruction of any of these vitamins, although a suggestion of a correlation with vitamin B_1 is observed in the effect of ultraviolet light on the rheobase and on the excitability of single nerve fibers (Hutton-Rudolph, 1943).

III. CHEMICAL CHANGES IN PROTOPLASM FOLLOWING IRRADIATION

Another problem occupying the center of interest at the present time concerns the nature of the chemical changes which occur in protoplasm after absorption of radiations. In photochemical systems absorption of ultraviolet radiations results in activated electronic levels which may cause ionization or dissociation, one energy-rich molecule resulting from the absorption of each quantum of light (Einstein-Stark law). This is known as the *primary reaction* and is followed by a *secondary reaction* or reactions. Some of the secondary reactions which occur in photochemical systems are: (1) resonance or re-emission at the same wave length, or fluorescence re-emission at longer wave lengths; (2) photosensitization or transfer of energy to other molecules, which may then react; (3) polymerization; (4) photolysis; (5) substitutions; (6) oxidations; (7) shift in equilibrium; (8) photosynthesis; and (9) intramolecular shift in bonds (see Dhar, 1931). As can be seen from Figure 12, the $Nh\nu$ values[7] for the ultraviolet spectrum are very large; therefore, even if energy is lost in transfers between molecules, sufficient amounts of energy are still available to drive most types of chemical reactions. It is unlikely that all of the above reactions occur in protoplasm, but there is some evidence for reactions Nos. 1, 2, and 7. Reaction No. 6 has also been considered as important by some workers.

With regard to reaction No. 1, some of the ultraviolet light absorbed is given off as visible fluorescence (Giese and Leighton, 1933); and the fluorescence of the skin, nails, cornea of the eye, teeth, etc., in the ultraviolet is a well-known phenomenon. Fluorescence with a long-time lag between absorption of light and its emission, called "phosphorescence" (Giese and Leighton, 1937; Harvey and Chase, 1942), occurs in cornified tissues and tissue products but not in active protoplasm, so far as we know. Only a small part of the light absorbed is emitted as fluorescence; the rest results in activation of molecules.

Photosensitization (reaction No 2, above) by transfer of energy (absorbed by a chromophore such as the purine or pyrimidine ring or the rings of aromatic amino acids) to other parts of the large molecules of proteins, probably occurs in cells. *In vitro*, such transfer of energy is known to occur in synthetic compounds from the aromatic ring to peptide bonds (Carpenter, 1940).

The earlier investigators believed that ultraviolet light produces its effects by reaction No. 6, i.e., by increasing the rate of various oxidations occurring in the cell, thereby burning up the vital compounds and leading to death (Downes and Blunt, 1877). If this were true, there should be a measurable increase in oxygen uptake following irradiation of cells. However, tests made by using the standard Warburg technique with yeast, luminous bacteria, ciliates, molds, and

[7] Where N is Avogadro's number, 6.06×10^{23} molecules per mole, and $h\nu$ is the energy value of a quantum in calories; h is Planck's constant, 6.624×10^{-27} erg/sec, and ν is the frequency of light equal to c/λ, where c is the speed of light, 3×10^{10} cm/sec, and λ is the wave length in centimeters (Stuhlman, 1943).

eggs and sperm of the sea urchin generally show little change in exogenous respiration following lethal doses of ultraviolet radiations (Giese, 1941*a*; 1942*a*).

In such studies the measurements were made in the same quartz vessels before, during, and after irradiation; therefore, even a small change could be observed. In a few cases a brief stimulation in respiration was found, usually followed by a retardation. Ultimately, in all cases the

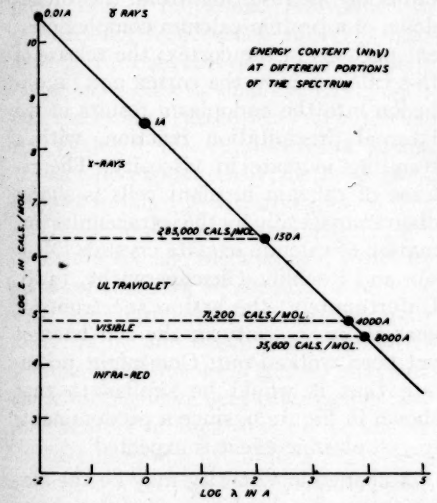

FIG. 12.—Energy content for different portions of the spectrum.

rate of respiration falls (Fig. 13), since cells so treated are unable to grow and replace the necessary constituents (see also Weintraub, 1944).

The fact that ultraviolet radiations are just as potent in their action even in the absence of oxygen is further evidence against the above theory. When a suspension of bacteria is freed of oxygen by bubbling hydrogen or nitrogen through it, care being taken to free the gases of oxygen, the bacteria are none the less killed by the radiations (Bie, 1905*b*) and killed just as readily as controls. It is probable that traces of oxygen are present in the cultures at the end of the period of preparation, but presumably these would be rapidly used up by the bacteria. Similarly, when the air is evacuated from a culture of bacteria, the radiations are no less effective (Wells, 1929). In this respect, the action of ultraviolet radiations resembles that of X-rays which are equally as potent in the absence of oxygen as in its presence but differs from the photodynamic action of visible light in the presence of certain photosensitizing dyes, which ceases when oxygen is re-

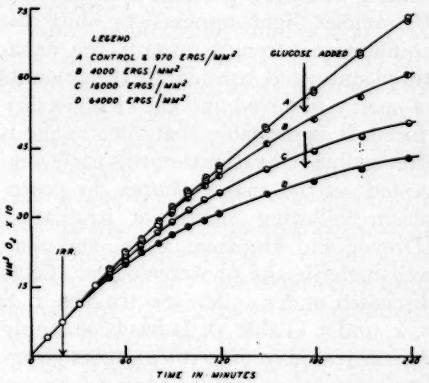

FIG. 13.—Retarding effect of ultraviolet radiations on exogenous respiration of luminous bacteria (Giese, 1941*a*, p. 210).

moved (Ledoux-Lebard, 1902; Blum, 1941, chap. vi). In the latter case, as might be expected, the uptake of oxygen is in some cases enormously accelerated by photodynamic action; and the R.Q.[8] may fall to 0.026, indicating that little carbon dioxide is being given off in comparison to the great oxygen uptake. The respiration in this case is insensitive to cyanide and may even be increased by it, indicating that the enzymes concerned in vital oxidations have little to do with the photodynamically accelerated oxidations. This

[8] R.Q. = respiratory quotient, or ratio of carbon dioxide given off to oxygen taken up.

is proved by bringing the cells to the boiling-point, for this does not prevent the oxidations from continuing. These photo-oxidations are therefore extraneous to the ordinary enzyme systems of the cell (Wohlgemuth and Szorenyi, 1933; see also Blum, 1941).

Reaction No. 7—shift in equilibrium by absorption of ultraviolet radiation—might be considered next. Denaturation of proteins is a reversible reaction (Anson and Mirsky, 1934), and an equilibrium between the native and the denatured state occurs wherever proteins are present. Ultraviolet light appears to shift the equilibrium strongly toward the denatured state; and, considering the amounts of energy involved and the changes produced, it is probable that the change is irreversible. Denaturation was early suggested as the major change in protoplasm following ultraviolet irradiation (Dreyer and Hanssen, 1907) and may well underlie the photobiological effects discussed under action spectra Nos. 1, 2, 3, 4, and 5 (Table 3). It has been amply demonstrated *in vitro* for proteins (Duggar, 1936, chap. viii) and is to be expected in the protoplasmic system where the main structural material is proteinous. Following denaturation, one might expect interference with the normal course of biological reactions. The interference depends on the portion of the protoplasm absorbing and being denatured —e.g., membrane, nucleus, or cytoplasm. The interference might be due merely to a physical change, such as a change in viscosity, or to the destruction of specific chemical catalytic elements, such as enzymes in the cells. In regard to the first possibility, increases in viscosity were early observed by Tschakhotine (1921; 1935), who irradiated a sea-urchin egg with a thin pencil of ultraviolet light and observed the decrease in Brownian movement of the particles. Heilbrunn and his co-workers, using the centrifuge technique, found slight decreases in viscosity after short irradiation, followed by increases after a greater dosage.[9] In *Arbacia* the increase is three- to fourfold (Heilbrunn and Young, 1930). Similar results were obtained with amebae (Heilbrunn and Daugherty, 1933). These viscosity changes do not occur if the calcium is first removed. Heilbrunn explains the increase in viscosity after irradiation as resulting from the breakdown of a protein-calcium complex present perhaps in the cortex; the release of this calcium from the cortex and its diffusion into the endoplasm results in the internal precipitation reaction, with a resulting increase in viscosity. The release of calcium in plant cells is shown diagrammatically by the intracellular formation of calcium oxalate crystals (Nadson and Rochline-Gleichgerwicht, 1928). Unfortunately, the action spectrum for changes in viscosity in the cell has not yet been worked out. One might postulate that it would be similar to that shown in Figure 6, since a predominantly cytoplasmic effect is expected.

A change in viscosity may be the major change involved in some of the effects of ultraviolet light showing an action spectrum resembling absorption by cytoplasmic proteins (Table 3, No. 3, also Fig. 6). The first effect of ultraviolet light on paramecia and other motile forms is a stimulation to greater activity, possibly corresponding to an initial decrease in viscosity. The stimulation of muscle fibers and the stoppage of protoplasmic streaming in *Nitella*, and the initiation of a propagated action potential as a result of very short flashes of ultraviolet light recently studied by

[9] Reed and Whitaker (1941) have recently described an interesting polarized plasmolysis which seems to be due to an increase in viscosity of the irradiated half of the egg.

Harvey (1942), might correspond to such a slight decrease in viscosity. After this initial stimulation, motile organisms gradually slow up and finally become immotile, showing only a few jerky movements; ultimately they vesiculate and cytolyze. The gradual slowing-up and immobilization may correspond to the increase in viscosity as denaturation proceeds.

It is doubtful if the effects—such as production of mutations, retardation of cell division from which recovery does not occur for several days, and complete cessation of growth—having action spectra resembling absorption by nucleic acid (Table 3, No. 1, and Figs. 2, 5, and 11) can be explained by mere viscosity changes induced by denaturation. These effects are so persistent that inactivation of certain specific biocatalysts, either by virtue of selective absorption or because they are more sensitive, seems more likely. Molecules occupying a "sensitive volume," centrosomes, genes, enzymes, and vitamins have been discussed as possible centers of such destructive action.

The conception of killing a cell by the absorption of a single quantum by a sensitive spot about the size of a protein molecule has been used in accounting for the bactericidal effect. When the logarithm of the survival ratio is plotted against the dosage, a straight line from the beginning of the experiment is thought to indicate a "single-hit-to-kill" (e.g., Wyckoff, 1932). This explanation has been attempted even for so large a cell as *Euglena* (Swann and Del Rosario, 1932), but it can hardly be invoked to explain the cumulative sublethal action of ultraviolet found in the case of animal cells.

Schleip (1923) tried to irradiate the centrosome selectively with a pencil of 2804 A and showed that the retarding effect on division was much less than when the nucleus was similarly irradiated, indicating no special sensitivity of the centrosome. Genes must serve as centers of action when mutations are induced by ultraviolet rays, since various facts make it seem unlikely that the mutation occurred because of a change in the immediate environment produced by the radiations (Stadler and Uber, 1942). It may be that in other cases where genetic evidence is lacking gene inactivations may, nonetheless, underlie the destructive changes observed after irradiation.

Vitamins of the B complex acting as prosthetic groups for respiratory enzymes might be selectively destroyed by ultraviolet light, since they absorb strongly in the lethal range; but there is no evidence for this yet. Mixtures of such vitamins were added to irradiated yeast in which the exogenous respiration was reduced below the control, but in no case was there any sign of recovery from the destructive effects of the radiations (Giese, unpublished).

The possibility of selective enzyme destruction has been an attractive hypothesis. Burge (1916) thought that perhaps the hydrolytic enzymes were selectively destroyed; but when he tested gelatin-liquefying bacteria after exposure to lethal dosages, he found that the enzymes still had their full activity. In luminous bacteria the luminescence was found to be more sensitive than the general respiration (Giese, 1941*a*, Fig. 3), but an analysis of the curves for luminescence decay after exposure to ultraviolet light (*ibid.*, Fig. 2) can be best interpreted by assuming an effect mainly on the substrate luciferin rather than on the enzyme luciferase. This fact is of itself interesting and indicates that at least this oxidative enzyme is not especially sensitive to the radiations. Yet, *in vitro* a similar enzyme from *Cypridina*

was readily inactivated by ultraviolet radiations (Chase and Giese, 1940). The enzymes are probably protected by protoplasm when in the cell and so are not selectively destroyed. Certainly, the total respiration of luminous bacteria and yeast given a lethal dose (Giese, 1941*a*, 1942*a*) does not decline until after a considerable lapse of time. The delayed decline might be explained as a failure to rebuild the catalysts which have been gradually worn out. One criticism may be leveled at the above statement, namely, that total respiration does not tell anything about the ways in which the reactions occur. Poisons—azide, for example—may even stimulate the rate of respiration but may, at the same time, prevent division of luminous bacteria, indicating interference with reactions which enable the organism to utilize the energy liberated (Giese, unpublished). Ultraviolet light does not stimulate total exogenous respiration, but it may greatly increase the endogenous respiration of starved yeast (e.g., Fig. 14), causing a two- to fourfold increase, which may last for as long as ten hours (Giese, 1942*a*). The excess endogenous respiration[10] induced by radiation is not extraneous to life, since bringing the cells to the boiling-point stops respiration. The respiratory quotient of the accelerated endogenous respiration is just about equal to the control. This indicates that the same substrate is being burned in both. The accelerated respiration is as sensitive to azide and cyanide as is the control (Giese, unpublished).

These experiments are of interest here because they show that certain oxidations may be accelerated by ultraviolet light, even if all the oxidations are not, and that this acceleration may be masked when the exogenous respiration is studied and no increase in total oxygen consumption is found. The experiments indicate a derangement in the respiratory activity resulting from irradiation which may be selective, in so far as it interferes with certain synthetic activities. An example of such a change is the loss of ability of yeast to remove glucose from the medium after irradiation. The fate of the glucose so removed has not been determined, but it is generally assumed that it is stored as carbohydrate in the cells. Ability to produce alcohol has also been found to decrease at the same time (Giese, unpublished). The action spectra for these effects have not yet been determined; therefore, no correlation with the absorbing substance can be made. The enzyme or enzymes involved might be destroyed either by inactivation of the prosthetic groups or by the denaturation of the protein-carriers.

Denaturation may underlie the photobiological effects involved in the activation of eggs, hemolysis, and erythema (Table 3, Nos. 3, 4, and 5; also Figs. 7 and 8). More direct evidence is, however, necessary in these cases.

Since denaturation plays an important part in at least some of the responses of cells to ultraviolet radiations, it may be worth while to review briefly the salient features concerning protein denaturation *in vitro*. The process occurs in at least three steps: (1) light alteration of the molecule; (2) chemical change developing the light-effect and rendering the molecule insoluble; and (3) aggregations of the molecules to form a flocculum (Clark, 1935, 1939). Step No. 1 can be shown to occur separately from No. 2 by irradiating a solution at low temperatures, at which step No. 1, being a photochemical reaction, will occur but step No. 2,

[10] It was not possible to demonstrate in the above case a diffusable respiratory accelerator, although Sperti, Loofbourow, and Dwyer (1937) present evidence that such an extract can be obtained from yeast if the cells are killed slowly by ultraviolet light or by other agencies.

being a thermochemical reaction, cannot occur. Raising the temperature will now result in step No. 2, rapidly followed by step No. 3. Step No. 2 can be shown to be separate from step No. 3 by irradiating pure protein to one side of the isoelectric point in the absence of salts. Then step No. 1 will depend upon the dosage, step No. 2 will vary in rate depending on the temperature, and step No. 3 will occur only on properly changing the pH.

When reversible denaturation occurs, as from mild heating, the linkages involved are probably secondary bonds (Anson and Mirsky, 1934). In the irreversible denaturation following ultraviolet light, other changes may well occur. Carpenter (1940) has shown that the carbon to nitrogen bond in dipeptides can be broken by ultraviolet rays. His experiments demonstrate that the chromophore can transfer the absorbed energy across several carbon bonds. The appearance of proteoses and peptones and even free amino acids in irradiated protein solutions indicates that the same may occur in proteins. Even more drastic changes than this may occur, for the characteristic band at 2800 A disappears after prolonged irradiation, indicating that the chromophoric rings are altered or destroyed. The appearance of ammonia is further evidence of photolysis (Arnow, 1936).

There is ample energy in quanta of ultraviolet light to disrupt such bonds (see Fig. 12). Approximately 71,000 calories are available per $Nh\nu$ of 4000 A, and double this at 2000 A. The energy required to break a carbon to carbon bond is estimated to be 58,600 cal/mole; a carbon to nitrogen bond, 48,600 cal/mole; and a carbon to carbon double bond, 100,000 cal/mole (Pauling, 1939). The utilization of the photochemical energy absorbed involves transfer across one or more bonds, with consequent loss; but transfer does occur, as already pointed out. However, drastic changes in the protein may not be necessary to alter the catalytic properties (Anson and Mirsky, 1934; Chase and Giese, 1940).

Denaturation of proteins does not explain the changes involved in tanning or in the prevention of neural-fold formation, and possibly not the hemolysis or

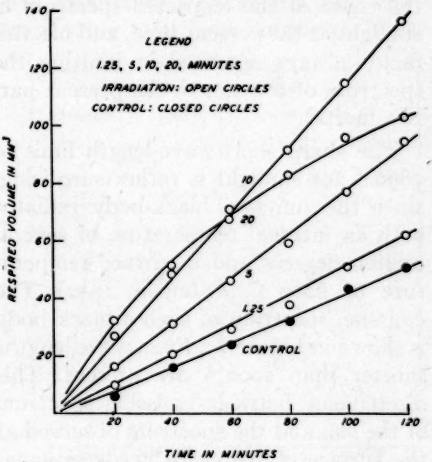

FIG. 14.—Stimulating effect of ultraviolet radiations upon endogenous respiration of yeast (Giese, 1942*a*, p. 38).

activation; and nothing more can here be added to what has already been said about these. However, these examples emphasize the point that the effects of ultraviolet radiations are probably always multiple; and when we isolate one or another criterion for study, we find one or another chemical agent responsible for that effect, but not necessarily for all the effects. This should be borne in mind in any analyses made.

IV. THE POSSIBLE ROLE OF ULTRAVIOLET LIGHT IN THE ORIGIN AND EARLY EVOLUTION OF LIFE

Finally, a problem of a speculative nature which has interested photobiologists

is whether ultraviolet radiations have played a role in the origin and early evolution of life. We have seen that sunlight has a rather limited spectrum in the ultraviolet, with a sharp cutoff at 2890 A. From what has been said about action spectra, the most potent ultraviolet radiations are of shorter wave lengths than those now represented in sunlight. The questions therefore arise: What is the cause of the restricted spectrum in sunlight at the present time, and has this factor always operated in limiting the spectrum of the sun to the present narrow limits?

The sharp short-wave-length limit of 2890 A for sunlight is rather surprising, since the sun is a black-body radiator with an internal temperature of several million degrees and a surface temperature of 6000° C. (Stetson, 1942). The emission spectrum of such a black body is shown in Figure 15. Even wave lengths shorter than 2000 A are present. This discrepancy between emission spectrum of the sun and the spectrum observed at the surface of the earth needs explanation. The earth's atmosphere is interposed between the sunlight and the surface of the earth. Experimental work on the atmosphere has demonstrated an ozone layer about 20 miles high in the atmosphere (Fabry and Buisson, 1930; Stetson, 1942), in the formation of which the short and deadly ultraviolet rays of the sun are filtered out. Oxygen absorbs strongly the wave lengths shorter than 2000 A; somewhat less strongly, the region from 2000 to 2530 A. The oxygen activated by absorption of the light forms ozone. Ozone absorbs continuously wave lengths shorter than 3000 A. Maximal absorption occurs at 2470 A. Thus the oxygen and ozone together screen out most of the radiations shorter than 3000 A and are the cause of the limited spectrum shown by sunlight at the present time.

The second question—whether the ultraviolet spectrum reaching the surface of the earth was always so limited—cannot be answered decisively. Hutchinson (1944), in reviewing the matter, states that some of the prominent authorities on biogeochemistry postulate the presence of oxygen in the early atmosphere during the cooling state of the earth; but another group of authorities believes that there is adequate evidence of a primeval earth devoid of oxygen or nitrogen in the atmosphere, the gases having been lost because the hot earth was unable to hold them. The only nitrogen remaining was that in bound-form in the rocks. Oxygen was bound in water and in oxides in rocks. The atmosphere present was largely made up of hydrocarbons and water vapor (Oparin, 1938), neither of which absorbs much in the region from 2000 to 3000 A. As evidence, it is pointed out that old geological formations take up oxygen avidly when exposed to the air, indicating their being in a reduced state, and that fossil carbon is present in excess over the total oxygen.

A fair probability, therefore, exists that the early atmosphere was devoid of oxygen. Since the remaining gases absorb but little in the short ultraviolet, the radiations reaching the surface may have been far more potent than today. In fact, these photochemically active radiations may have activated the inert masses of chemicals dissolved in the seas first to form complex chemical compounds of the organic type and then to form the first primordia of life itself. Photochemical production of carbohydrates from carbonic acid and water is said to occur in the ultraviolet (Baly, 1929). Photochemical activation of this type avoids some of the pitfalls of thermal activation so often postulated.

Such short radiations would continue to act upon the primordial protoplasm in another way. The gene mutations produced by ultraviolet light have been discussed (Stadler and Uber, 1942), and it was pointed out that radiations shorter than those found in the present sunlight are most effective. The short radiations during the early history of the earth would have accelerated the rate of mutation, thus providing the material for selection; and if one accepts the theory of natural selection, this would have accelerated the rate of evolution.[11]

Oparin thinks it logical, on the basis of the various facts which he enumerates in his book (1938), to postulate that the primary organisms appearing in a world characterized by a reduced state were anaerobes. Next came photosynthetic organisms, the earliest of which probably did not use water as a hydrogen donor and therefore did not liberate oxygen as a by-product (see in this connection van Niel, 1941; Franck, 1941; Blum, 1937). Finally, when water was used as the hydrogen donor, oxygen was liberated as a by-product. With the release of oxygen into the atmosphere the evolution of aerobic life began; and it gained expression to become the dominant type of life, bearing with it the capacity for a certain degree of anaerobic existence, sometimes large, sometimes small—a relic of the anaerobic past.

Until the oxygen appeared in the atmosphere, the death-dealing short ultraviolet radiations must have made life a hazard, which could be maintained only in the shadow of rocks or of other objects opaque to these radiations. Water, while somewhat opaque (Dawson and Hurlburt, 1934), would be a protection only if very deep. In fact, the presence of these radiations in sunlight beyond our atmosphere renders it unlikely that the germs of life in the form of cysts or spores could have drifted to the earth from some other planet, for they would have perished from the absorption of these radiations even if they could have survived the many other hazards along the way. This theory at one time had the support of a number of outstanding men (see Becquerel, 1910, for a discussion); and Lipman (see Oparin, 1938) has recently claimed isolation of bacteria from meteorites, a claim not generally accepted (Oparin, 1938) and laid to faulty techniques.

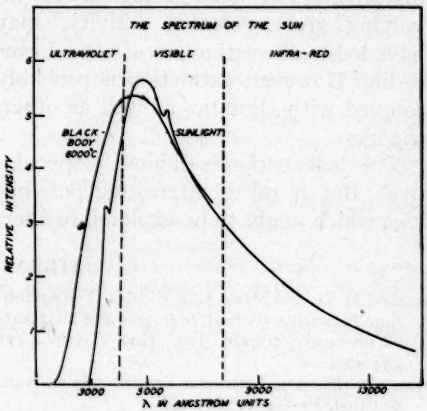

FIG. 15.—The sun's spectrum (from several sources).

With the appearance of the ozone umbrella subsequent to the development of organisms liberating oxygen, the powerful actinic radiations of sunlight were no longer able to penetrate to the earth, except for radiations of 2890–3000 A, which are present in the sunlight of today and produce sunburn, the bactericidal effect, etc. This change in the nature of sunlight reaching the surface of the earth may help explain the fact that, while life may have originated by spon-

[11] Since these short radiations are strongly absorbed by the most superficial layers of cells, they are not very likely to produce mutations in large organisms.

taneous generation at one time, there is no evidence, to the best of our knowledge, for such occurrence at the present time. If the radiations were the only source of energy for the reactions culminating in life, the process could not repeat itself, even if other necessary conditions remained favorable, once the ozone layer appeared.

Marshall (1928) has emphasized the possibility that decreases in ultraviolet light, such as occur during catastrophic disturbances between geological eras involving great volcanic activity, may have led to the extinction of some forms of life. However, extinction is probably coupled with climatic, as well as other, changes.

This last section is obviously speculative. But it raises interesting possibilities which ought to be explored further. Even if future research renders less probable the view that the atmosphere of the earth in its early history transmitted the short ultraviolet, interest in ultraviolet radiations will not diminish because determination of the action of sunlight as we know it today depends upon studies with short radiations. Furthermore, the action-spectrum method, involving wave lengths shorter than those of sunlight, may be used as a means of analysis of other biological problems, as has been attempted in the case of neural-fold formation. Should future researches render more probable an early atmosphere which transmitted the very short sunlight radiations, the interest will redouble, since the origin of life is one of the most interesting of all the problems which confront not only the biologist but every man.

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OXIDATION-REDUCTION PATTERNS IN CILIATE PROTOZOA

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SEVERAL studies of differential reduction of methylene blue and other vital dyes by *Paramecium* have been made, but in most of them (Kalmus, 1928; Roskin and Semenoff, 1933; Gersch, 1937) conclusions wholly or in part incorrect concerning the reduction pattern have been reached. The incorrect conclusions result from failure to distinguish the gradient patterns of uninjured animals from those modified or reversed by differential toxic effects of the dyes or of reagents used to obtain the reduced forms, the so-called "leucobases," of the dyes. Experiments with a wider range of concentrations of dyes and of reducing agents would have avoided these errors, as Child (1934) pointed out. Nevertheless, some of the errors appear in the paper by Gersch (1937). In view of this situation, further data on oxidation-reduction patterns in *Paramecium* and other ciliates have seemed desirable. Also, an extended study of intracellular formation, reduction, and reoxidation of indophenol in living *Paramecium* and a number of other ciliates provides another line of evidence concerning pattern for comparison with the patterns shown by the dyes. In earlier work, brief mention was made of the pattern of indophenol reaction in living paramecia (Child and Deviney, 1926, pp. 294–96), but except for this mention the data on indophenol patterns in the present paper apparently constitute the only record of observations on living protozoans with this method.

The earlier study of pattern in *Paramecium* with methylene blue has now been supplemented by use of three other oxidation-reduction dyes—Janus green (diazine green), Nile blue sulphate, and neutral red, which has also been used as pH indicator—and some further data have been obtained with methylene blue. With all of these, intracellular reduction of the oxidized dye and oxidation of the leucobase have been observed in a wide range of concentrations. Use of the different dye concentrations and of a reducing agent, virtually nontoxic in the concentrations required, have made it possible to distinguish with certainty the pattern of the "normal" uninjured individual from modifications of pattern due to toxic effects. Photodynamic effects and the effects of cyanide on pattern have also received attention.[1]

[1] I am indebted to Dr. E. L. Tatum, of the Stanford University School of Biological Sciences, for discussion and suggestions concerning certain points; but he is not responsible for conclusions or interpretations appearing in this paper.

MATERIAL AND METHODS

The forms most extensively studied are *Paramecium multimicronucleatum*, a species of *Frontonia*, and *Spirostomum teres*. The *Paramecium* material was kindly supplied from laboratory stock by Dr. W. H. Johnson; *Frontonia* and *Spirostomum* were collected from localities near Palo Alto. *Frontonia* resembles *F. leucas* in form; but it is entirely without zoochlorellae and has a less clearly marked, i.e., less steep, gradient pattern, than the *Frontonia* material of the earlier paper (Child, 1934). Because *Paramecium multimicronucleatum* shows a very distinct gradient pattern, reacts rapidly with the indophenol reagents, stains, reduces, and reoxidizes readily, and is not extremely susceptible to toxic action, it has been used more extensively in certain lines of experiment than the other forms. Observations on indophenol pattern have been made on various other ciliates as opportunity offered.

Yeast cultures provided abundant material of the three forms, new cultures being started as desired, using a few pieces of yeast from a yeast cake in water, and inoculating from old cultures. All three forms were grown in some of the cultures so that they could be directly compared in the same experimental lot. *Frontonia* and *Spirostomum* became very numerous in the older yeast cultures with repeated additions of yeast. *Frontonia* ingested in large numbers the minute ciliates which commonly appeared in the cultures. *Paramecium* multiplied most rapidly in new cultures a few days old but was also present in the older cultures. No pure-line cultures were made. Data presented on the three species are based on more than a thousand experimental lots, many of them containing all three. Intracellular formation of the brilliant blue indophenol from para-aminodimethylaniline (para-dimethylphenylenediamine) and α-naphthol, both in highly dilute aqueous solution; intracellular reduction and reoxidation of indophenol; staining, reduction, and reoxidation of the oxidized dyes Janus green, Nile blue sulphate, neutral red, and methylene blue; and primary intracellular oxidation of the leucobases can all be observed in living, normally active animals, reduction and reoxidation often repeatedly in the same individual.

Indophenol, neutral red, Nile blue sulphate, and methylene blue become colorless on intracellular reduction and regain color on reoxidation. Janus green undergoes two color changes in the usual reduction: first to a brilliant red on partial reduction and then from red to colorless on further reduction. The first change to red makes this dye particularly useful in bringing to light slight regional differences. When reduced by sodium hydrosulphite (see p. 253), neutral red first becomes colorless and then, with sufficient hydrosulphite, lemon yellow, although the solution is acid after the addition of hydrosulphite. This yellow form is completely reversible on oxygen increase.

Intracellular formation of indophenol is catalyzed by an oxidase, at present believed to be cytochrome oxidase. According to current views, the flavoprotein-dehydrogenase system is concerned in reduction of the indicators. Intracellular reoxidation of reduced indicators is again catalyzed by oxidase. Intracellular formation of indophenol from the two reagents is often referred to in the following text as the "indophenol reaction," or in certain contexts as "the reaction."

Several earlier papers have been concerned with intracellular formation, reduction, and reoxidation of indophenol in various living organisms. Procedures employed with living material were most

fully described in one of them (Child, 1944*a*), and attention was called in that paper and in a special note (Child, 1944*b*) to an error in preceding papers concerning concentrations of para-aminodimethylaniline used with the living material. Stock solutions of aniline were prepared, as described in the paper cited above, one drop of liquid aniline (40 drops = 1 ml.) in 10 ml. of water and 1 mg. of α-naphthol (weighed or estimated) in 10 ml. of water. For use these solutions were further diluted from 5 to 500 times—for certain purposes, even more—with culture water containing the animals. The highest concentrations of this range are rapidly toxic and lethal, the lowest give the reaction only partially. Between the extremes there is a wide range of concentrations, absolute and relative, of both reagents in which the same reaction pattern appears more or less rapidly. For convenience one small drop (40 = 1 ml.) of the stock solution of each reagent in 1 ml. of culture is regarded as standard concentration and is so designated in the following text.

Wide ranges of concentration of the dyes Janus green, Nile blue sulphate, neutral red, and methylene blue were used. Janus green and methylene blue, 1/10,000 in dechlorinated tap water, which was also used in the cultures, served as stock solutions. For staining, Janus green 1/800,000–1/100,000 was used; 1/100,000 and higher concentrations of the oxidized dye are rapidly toxic and lethal to *Paramecium* and *Frontonia; Spirostomum* is somewhat less susceptible. Methylene blue, being much less toxic, was used in concentrations from 1/200,000 to 1/10,000. Stock solution of the highly toxic Nile blue sulphate was 1/100,000, and useful concentrations for staining ranged from 1/2,000,000 to 1/200,000. Neutral red concentrations in tap water or culture water are uncertain because crystal formation begins in a few minutes. Concentrations initially 1/500,000–1/20,000 in culture water from stock solution 1/10,000 in distilled water were used in most cases; but for certain comparative experiments in which exact concentrations were not important, small amounts of neutral red were dissolved in culture water and diluted as desired for immediate use. For extreme cytolytic effects higher concentrations than for oxidation-reduction were used up to those in which cytolysis begins almost immediately. In order to distinguish with all possible certainty the oxidation-reduction patterns of uninjured animals from patterns altered by toxic effects of the dyes, many concentrations of each dye were used under varied experimental conditions. Also, the most satisfactory concentrations for a given purpose differ considerably with the three ciliates, often with different cultures and with different individuals of a single experimental lot from a single culture. However, the general oxidation-reduction patterns are so definite, so clearly visible, and so readily reproducible that it is not difficult to find satisfactory working ranges of concentration; and very considerable differences in concentration do not obscure or essentially alter the pattern. Different samples of aniline obtained from the Eastman Kodak Company were found to vary considerably in effect. Some were more toxic than others, and in a wide range of concentrations the animals cytolyzed without showing deep color or strongly marked gradient pattern. Even the most satisfactory samples finally became more toxic, and only slight gradient patterns appeared. The samples may be solid or liquid; if solid, they become liquid on slight warming.

Intracellular reduction of indophenol and the dyes may be brought about

slowly by oxygen decrease in the solution, resulting from oxygen uptake of the animals when considerable numbers of individuals are sealed in a small volume of solution or often when they are merely brought under a cover glass. In aggregations of *Paramecium* and *Frontonia* in open solutions oxygen decrease is often sufficient to bring about more or less reduction. Intracellular reduction of all the indicators may be brought about as rapidly as desired, even within a few seconds, by the addition of a minute amount of sodium hydrosulphite ($Na_2H_2S_2O_4$) to the solutions containing the animals, oxygen decrease resulting from the rapid oxidation of this agent. A very small fraction of a milligram, consisting of a few minute granules, is sufficient for reduction in 1 ml. of solution; and much larger amounts are not appreciably or not rapidly toxic. Hydrosulphite also makes it possible to obtain the reduced forms, the so-called "leucobases," of oxidation-reduction dyes in a few seconds at any time and to use them for staining in order to determine whether a catalyzed oxidation pattern appears in the animals. The leucobases of all the dyes used are much less toxic and less irritating than the oxidized dyes, but they may become toxic on oxidation much more rapidly than the same concentrations of oxidized dyes because the leucobases enter and accumulate within the cells much more rapidly than the oxidized forms. Sodium hydrosulphite as a reducing agent is far more satisfactory than the rather highly toxic reducing agents used in earlier work. Those agents may alter oxidation-reduction patterns and, in absence of adequate controls, have led several investigators to erroneous conclusions.[2]

After reduction, reoxidation with return of color in the still normal active animals can be brought about by exposure of the sealed preparation to air, by aeration of a solution reduced by hydrosulphite, or gradually by diffusion of oxygen into the solution open to air without agitation. However, both slow and rapid oxygen increase are of interest, for the direction of progress of intracellular reoxidation with slow oxygen increase is the reverse of that with rapid increase.

With the lower indicator concentrations or after slight reaction or staining, the color may be less deep after reoxidation than originally, particularly in *Paramecium*. As will appear below, intracellular accumulation of the indicators apparently increases the reducing power of the animals; also, certain observations suggest that the animals are able gradually to destroy or alter irreversibly with decoloration low intracellular concentrations of the indicators. This question will be dealt with later.

Incidentally, it may be noted that indophenol solution oxidized *in vitro* acts as a relatively weak stain, either in the oxidized form or after reduction with intracellular reoxidation and, if depth of color at time of death is an adequate criterion, is more toxic than indophenol produced by intracellular reaction. However, if toxicity is not too great, intracellular reduction may occur in low oxygen.

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[2] Kalmus (1928) used $Na_2S_2O_3$; Roskin and Semenoff (1933) and Child (1934) used HCl and $Na_2S_2O_3$; and Gersch (1937) used rongalite, a formaldehyde-sodium hyposulphite agent for reduction of dyes. These agents are all toxic. Child (1934) showed that the differential toxic effect of a reducing agent or of too deep staining may alter or completely reverse the reduction pattern and that the reduction patterns of *Paramecium* observed by Roskin and Semenoff were reversals of the normal pattern, resulting from differential toxic effect of reducing agent or dye. Later, however, a similar failure to distinguish alteration of reduction pattern by differential toxic effect appeared in Gersch's paper.

Oxidation-reduction has been studied in its relation to fission, particularly as regards the development of the anterior end in the posterior member of a fission-pair and the difference in condition of anterior and posterior members in earlier and later stages and in some cases after separation. Some observations on photodynamic action of the indicators and photodynamic effects on pattern have been made. Otherwise indicator preparations have been kept in darkness or very dim diffuse light, except during microscopic examination. If cyanide inhibits oxidase activity, oxidation-reduction indicators may be expected to give some evidence of its action. Potassium cyanide has been used in a wide range of concentrations and exposure periods with interesting results.

It is scarcely necessary to point out that figures drawn in black ink are of very limited value as a means of indicating the color gradations and progressive changes with indophenol, in primary oxidation of leucobases, and in reduction and reoxidation of dyes. Only large numbers of colored figures would serve to show these in some measure. Even if movement can be completely prevented, e.g., by methyl cellulose, photomicrography presents many practical difficulties with the living animals and at best can show only certain periods rather than particular stages in the progress of the color changes; for reduction by hydrosulphite, intracellular oxidation of leucobases, and reoxidation with rapid oxygen increase, all of which may be complete in a few seconds, it is of no value. Fixed material is quite unsatisfactory for photographic record. In an attempt to indicate in some degree the chief features of the patterns and their modifications, diagrammatic figures are included in the paper, with arrows to show direction of progress of color change and point shading to indicate roughly the gradations. But only by direct observation of the living animals can the delicacy and beauty of the gradient patterns and their modifications be adequately realized.

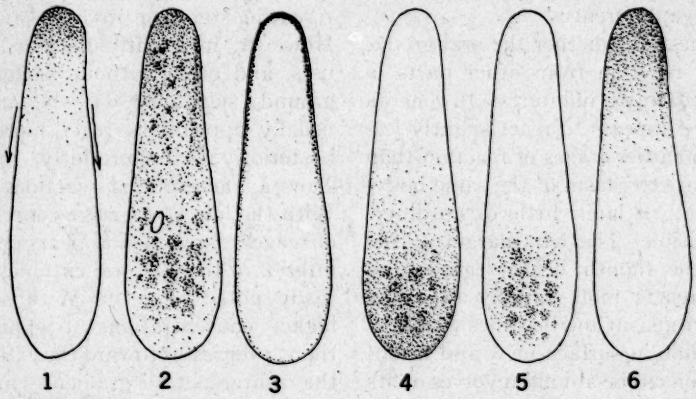
In describing the gradient patterns and their modifications convenient and adequate terms for indicating direction of progress of the differential color changes are desirable. The terms "posteropetal" and "anteropetal," though not in common usage, are entirely clear, more exact than "basipetal" and "acropetal," and more convenient than "from anterior to posterior," or the reverse, and will therefore be used.

PATTERNS OF INDOPHENOL REACTION, REDUCTION, AND REOXIDATION

Paramecium multimicronucleatum

Indophenol patterns and their modifications have been more extensively studied in *Paramecium* than in other ciliates. With concentrations of reagents ranging from one-fourth standard to three or four times standard, the characteristic indophenol reaction pattern in the ectoplasm is a posteropetal gradient (Figs. 1 and 2). In the higher of these concentrations the anterior ectoplasm usually becomes almost opaque with indophenol before cytolysis begins (Fig. 3). More posterior levels react less deeply. However, different lots and different individuals of the same lot vary considerably in rate of the reaction. Moreover, *Paramecium* reduces indophenol with so little oxygen decrease that even slight aggregations for periods of 2–3 minutes may bring about more or less posteropetal decoloration in at least some individuals. That this is reduction is evident, since recoloration occurs almost at once when the animals are dispersed in the solution by agitation, with a little aeration. In concentrations of reagents half

of standard or lower, the posteropetal ectoplasmic reaction gradient appears in the earlier stages of reaction; but, instead of progressive increase in color, a posteropetal decoloration gradient may begin to appear, quite independently of oxygen decrease, by aggregation or otherwise and even with frequent aeration or in a thin film of solution open to air. In still lower concentrations the reaction gradient may be anteropetal instead of posteropetal from the beginning of reaction; and most or all of the preoral region may remain almost or quite without color (Fig. 4), or color may appear only in the postoral entoplasm (Fig. 5). These modifications of pattern must be due either to a posteropetal gradient of decoloration of intracellular indophenol by reduction or to gradual posteropetal alteration or destruction of one or both reagents or of indophenol. An increase in respiration by para-aminodimethylaniline has been observed in some organisms. If this reagent increases respiration in *Paramecium*,[4] reduction of intracellular indophenol in higher oxygen content of the solution than before the increase may be expected until toxic effect appears. In some cases these anteropetal reaction patterns show posteropetal increase of color on aeration, indicating that the pattern was due, at least in part, to reduction. Other cases show no increase, even in high oxygen concentration; these suggest an irreversible decoloration of intracellular indophenol. Moreover, animals transferred from the solution of reagents to water before the reaction has become highly toxic show gradual posteropetal decoloration, not reversible by oxygen increase and without evidence of loss of indophenol to the water.



FIGS. 1–6.—*Paramecium:* indophenol and dye oxidation-reduction gradient patterns. Figs. 1 and 2, early and later stage of indophenol reaction; also of rapid reoxidation of reduced indophenol and dyes and staining gradient in solutions of high pH, except with neutral red. Fig. 3, optical section, indicating ectoplasmic indophenol reaction gradient, gradient of rapid oxidation of reduced indophenol and dyes, and staining gradient in solution of high pH, except with neutral red. Fig. 4, partial indophenol reaction with low concentrations of indophenol reagents; also a stage of posteropetal reduction of indophenol and dyes and of slow anteropetal oxidation of reduced indophenol and dyes. Fig. 5, indophenol reaction and staining with very low concentrations of indophenol reagents and dyes, limited to interior of postoral entoplasm; also a late stage of reduction and an early stage of slow oxidation of reduced indophenol and dyes and of reaction and staining in solutions of low pH. Fig. 6, early stage of indophenol reaction, indicating more rapid reaction along margin of oral groove.

Rapidity of reaction, as indicated by appearance and depth of color, differs with nutritive condition: starved animals react much less rapidly, but are more susceptible and die with much less reaction, than well-fed, rapidly dividing animals. Other unanalyzed cultural conditions are probably factors in determining that some cultures react more rapidly than others without definite evidence of nutritive differences.

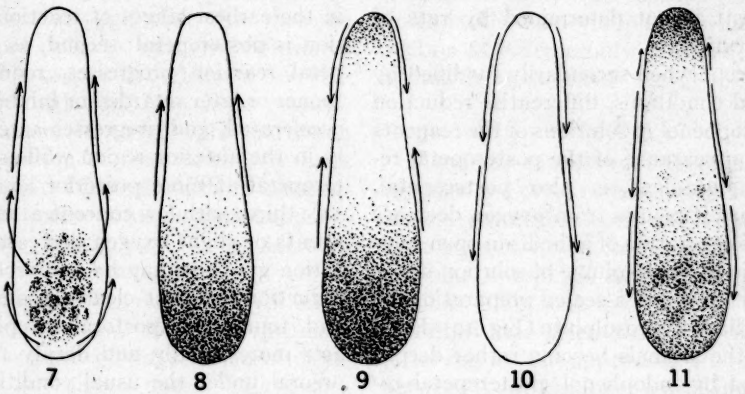
The question whether the oral groove differs in reaction from other parts in any definite way is of interest. In general, the groove appears to react slightly less rapidly in earlier stages of reaction than the general ectoplasm of the same body-levels (Fig. 2); later, little or no difference is visible. The left margin of the groove (the mouth being regarded as ventral) reacts more rapidly than the general ectoplasm and becomes visible as a darker line in surface view and in optical section as the animal revolves on its long axis (Figs. 2 and 6). In some cases the right margin of the groove also seems to react slightly more rapidly than the general ectoplasm, but this difference is so slight at best and so frequently not distinguishable that its reality is somewhat doubtful. In later stages of reaction, as toxic effects appear, the groove becomes flattened and indistinguishable from other ectoplasm. The groove shares in the general posteropetal reaction gradient of the ectoplasm. Whether its less rapid reaction in earlier stages results from less oxidase activity than elsewhere or from reduction or destruction of the very low indophenol concentrations in its ectoplasm in early reaction is uncertain. With some of the dyes it seems to reduce a little more rapidly than the general ectoplasm.

Since the entoplasm undergoes cyclosis, presence in it of a definite persistent gradient may seem impossible. However, recently formed food vaculoes are, in general, most numerous or only present in postoral entoplasm. Digestion is more advanced in preoral regions. Reaction is generally more rapid and color usually becomes deeper in postoral than in preoral entoplasm. This difference is due in part to more rapid reaction of recently formed food vacuoles or their contents and of other inclusions, presumably representing stages or products of digestion. However, in slightly flattened individuals, and often without flattening, the ground substance of the entoplasm usually appears to react more rapidly posteriorly than anteriorly, that is, to show an anteropetal reaction gradient. With the lowest effective concentrations of reagents, reaction appears only in the interior of the postoral entoplasm, as already noted (Fig. 5). With somewhat higher concentrations it appears first there, progresses toward the exterior, and the anteropetal gradient mentioned above gradually appears in the postoral ectoplasm (Fig. 7). No evidence has been obtained to indicate that intake of reagents by mouth is concerned in these patterns. If solution does enter by mouth and water passes to the contractile vacuole system, the concentration of reagents may become somewhat higher in the postoral entoplasm than elsewhere. However, this region is less able to reduce or destroy indophenol than any other. If there is a real entoplasmic reaction gradient, as there appears to be, at least under certain conditions, it is undoubtedly an expression of digestive and nutritive functions of the entoplasm and represents a change in condition in the entoplasm as it passes anteriorly in cyclosis with progressive alteration and probable loss to ectoplasm of some of its constituents.

With concentrations of reagents three, four, or more times standard, there is at

first considerable irritation. Many or all individuals move rapidly and continuously or almost continuously backward. In these intracellular indophenol is visible in the posterior ectoplasm within a few seconds and earlier than elsewhere, and the ectoplasmic gradient is completely anteropetal (Fig. 8); or in some individuals, with less extreme irritation, color appears at the anterior end somewhat later and served in earlier work with *Paramecium caudatum* when backward movement was induced by highly irritating concentrations (Child, 1934), and data presented below show reversal in leucobase oxidation of high concentrations of other dyes (p. 265).

Both indophenol reagents are more or less rapidly toxic and lethal in sufficient concentration. With exposure to one of



FIGS. 7–11.—*Paramecium:* indophenol and dye oxidation-reduction gradient patterns. Fig. 7, early stage of slow oxidation of reduced indophenol and dyes and of indophenol reaction and staining at low pH. Fig. 8, anteropetal indophenol reaction and oxidation of reduced dyes with continued backward locomotion under irritating conditions. Fig. 9, anteropetal and later posteropetal indophenol reaction and oxidation of reduced dyes with backward locomotion under less extreme irritation than Figure 8. Fig. 10, the posteropetal reduction gradient of indophenol and dyes in low oxygen; also with exposure to KCN after moderate staining. Fig. 11, reduction of indophenol and dyes with anterior differential retardation; also with KCN after deep staining.

the animals show two opposed reaction gradients (Fig. 9). The anteropetal gradient is rather steep; that is, the posterior region becomes deeply colored before the reaction progresses to the anterior end (Fig. 8). The anteropetal reaction in these high concentrations of reagents may extend over the whole body-length in one minute or less. These reversals of reaction pattern seem to indicate that oxidase activity is increased posteriorly in the backward-moving animals. A similar reversal of the oxidation gradient of the leucobase of methylene blue was obtained them just long enough for the earliest visible toxic effects (posteropetal retardation of cilia and slowing of locomotion, to appear in a few of the more susceptible individuals, before adding the other) the reaction may be completely anteropetal, in consequence of differential susceptibility of the ectoplasm to the first reagent; that is, a gradient of retardation of reaction, the effect decreasing posteriorly, has been brought about by the first reagent. This, of course, is a differential toxic effect of the reagent; but it may appear before there is any direct

evidence in form or behavior of toxic effect, except perhaps slightly less rapid movement in some individuals.

The modifications of the posteropetal reaction gradient indicate that it is not due merely to a posteropetal differential in permeability to one or both reagents. Moreover, exposure to one reagent for a short time before addition of the other shows that both penetrate all body-levels almost at once and that the reaction gradient is not determined by rate of penetration.

Except when secondarily modified by special conditions, differential reduction of indophenol in solutions of the reagents after appearance of the posteropetal reaction gradient is also posteropetal, whether it results from oxygen decrease in an aggregation of animals in open solution, in a small volume of solution under a cover glass, in a sealed preparation, or by sodium hydrosulphite (Fig. 10). Even after the animals become rather deeply colored by indophenol, posteropetal reduction to colorless is still possible. Entoplasm reduces less rapidly than ectoplasm but also posteropetally. Animals slightly flattened under a cover glass during reduction show a distinct posteropetal reduction gradient in the ground substance of the entoplasm. Here, again, the question whether a real gradient is present in the entoplasm arises. The possibility that posteropetal entoplasmic reduction may be a secondary result of posteropetal decrease in ectoplasmic oxygen uptake must be noted. In a medium low in oxygen the greater oxygen uptake in anterior ectoplasm may conceivably determine more rapid oxygen decrease, and so more rapid reduction in the anterior entoplasm and a posteropetal reduction gradient. On the other hand, this gradient, like the entoplasmic reaction gradient, may be an expression of progress in digestion and utilization of nutritive material with movement anteriorly of the entoplasm.

Since the region of most rapid ectoplasmic reaction normally becomes the region of most rapid reduction, it appears possible that the posteropetal reaction may alter the ectoplasmic condition by differential stimulation, irritation, or otherwise, so that a posteropetal reduction gradient results. As regards this question, it may be noted: first, that even in the earliest stages of reaction, reduction is posteropetal; second, as posteropetal reaction progresses, reduction is sooner or later retarded or inhibited, not accelerated, and progresses anteropetally in the anterior region while still posteropetal at more posterior levels (Fig. 11); third, with low concentrations or reagents or slight oxygen decrease the reaction gradient may be anteropetal, the reduction gradient clearly posteropetal; and, fourth, the postoral entoplasm reacts more rapidly and deeply than the preoral under the usual conditions but reduces more slowly.

After retardation by accumulated intracellular indophenol, anterior regions may still be able to reduce slowly in part or wholly with anteropetal progress of reduction, as indicated in Figure 11. According to degree of toxic effect of indophenol, reduction may finally reach the anterior tip, often after all other ectoplasmic levels are reduced; or it may stop short of the tip, which soon begins to cytolyze. With further progress of reaction the retardation extends farther posteriorly, and progressively less of the ectoplasm remains capable of normal reduction. Under these conditions the reduction gradient may become entirely anteropetal. Similar reversal of the methylene blue reduction gradient by differential toxic effect of the oxidized dye has been observed in *P. caudatum* (Child, 1934). It was failure to observe that the

anteropetal reduction gradient resulted from a posteropetal gradient of toxic effect of dyes or reducing agents that led Roskin and Semenoff (1933) and Gersch (1937) to incorrect conclusions concerning reduction.

Reduced intracellular indophenol is much less toxic than oxidized. Until affected by lack of oxygen *Paramecium* may remain normal in form and behavior, with intracellular concentrations of indophenol so high that they become toxic and initiate cytolysis within a minute or two, or even after a few seconds, on oxidation. Also, after retardation of locomotion and slight shortening and alteration of form anteriorly have begun as an effect of oxidized indophenol, some recovery may occur temporarily, and cytolysis may be considerably delayed by reduction.

Reoxidation with recoloration of intracellular indophenol following increased oxygen content of the solution after reduction by oxygen uptake of the animals may occur in a few seconds, or more slowly, according to rate of oxygen increase. With rapid increase by active aeration of the solution, reoxidation, like the reaction, usually progresses posteropetally in the ectoplasm; in the postoral entoplasm it is less rapid, but the color finally becomes deeper there. With more gradual oxygen increase—for example, by diffusion of oxygen from the surface into the solution—recoloration may show two opposed gradients or may be completely anteropetal. In the first case the reoxidation pattern resembles the normal reaction pattern (Figs. 1–3); with gradual oxygen increase the two opposed gradients are regionally similar to Figure 9, but the anteropetal gradient is less deep in color, and color appears in the postoral entoplasm; with completely anteropetal gradient, stages like Figures 4 and 7 occur. These differences suggest that, with rapid oxygen increase, the differential in oxidase activity may determine the posteropetal ectoplasmic gradient; with gradual oxygen increase, a gradient in oxygen uptake, decreasing posteriorly, may determine that oxygen concentration becomes high enough for reoxidation in the posterior region slightly earlier than anteriorly. With moderate concentrations of reagents reduction and reoxidation may be repeated several times in the same individuals until reduction is differentially retarded or inhibited by toxic effect.

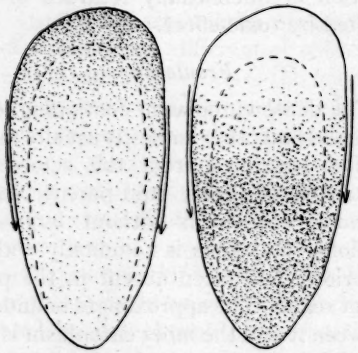
Frontonia

The dense entoplasm, containing food vacuoles and other bodies, is usually more or less separated from the ectoplasm by a zone of nearly transparent, finely granular entoplasm without large inclusions. This zone is commonly widest anteriorly and often absent in the posterior region. The approximate boundary between it and the inner entoplasm is indicated in Figures 12 and 13 by the broken line. In animals gorged with recently ingested food it may be absent, and during fission it is less conspicuous than at other times or absent. Food vacuoles and other inclusions usually become more numerous posteriorly in the inner entoplasm, with more or less debris, dark in color, at the posterior end. So far as observed, cyclosis appears to be rather an indefinite flowing in localized areas than a continuous, definitely directed movement of entoplasm; but the usual distribution of food vacuoles indicates some difference in condition between anterior and posterior regions of the inner entoplasm.

The ectoplasmic reaction gradient in *Frontonia* is posteropetal (Fig. 12). The inner entoplasm becomes deep in color, usually deeper posteriorly than anteriorly. *Frontonia* is more susceptible than

Paramecium to toxic effects of indophenol and the reagents, and in the same lot with *Paramecium* dies earlier. Concentrations of reagents considerably lower than those most satisfactory for *Paramecium*—for example, a third to a fourth of standard—give the best results.

Reversal of locomotion in high concentrations of reagents is less frequent and less continuous than in *Paramecium* and *Spirostomum*, usually alternating with periods of forward locomotion.



12 13

FIGS. 12 AND 13.—*Frontonia:* Fig. 12, posteropetal indophenol reaction gradient; also, rapid oxidation of reduced indophenol and dyes. Fig. 13, posteropetal indophenol and dye reduction gradient; slight posteropetal indophenol reaction gradient visible in posterior half.

However, in some backward-moving animals, slightly more rapid reaction in the posterior ectoplasm with an anteropetal gradient has been observed for a few seconds.

Until toxic effects appear, ectoplasmic reduction in aggregations, in sealed preparations, and with hydrosulphite is posteropetal (Fig. 13). Entoplasmic reduction is also posteropetal except in animals gorged with food, in which there is little regional difference. The inner entoplasmic zone reduces much less rapidly than the outer. Differential toxic effect retards reduction posteropetally and so may partially or completely reverse the reduction gradient. As in *Paramecium*, reoxidation is either posteropetal or anteropetal, according to rate of oxygen increase. In rapid, posteropetal reoxidation the anterior ectoplasm precedes other parts; in slower, anteropetal reoxidation, color appears first in the posterior entoplasm. No definite evidence of destruction of intracellular indophenol by *Frontonia* has been obtained.

Spirostomum teres

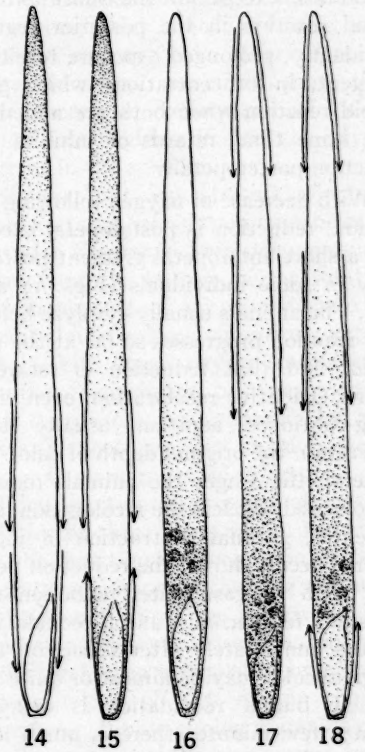
With all effective concentrations of reagents *Spirostomum* shows the indophenol reaction less rapidly and becomes less deeply colored than *Paramecium* and *Frontonia*. In the same experimental lot and standard concentrations of reagents, all or almost all individuals of *Spirostomum* live longer than the other forms. Different individuals show considerable differences in rate and pattern of reaction. In standard or somewhat higher concentrations of reagents, the reaction in some individuals is posteropetal over the whole length (Fig. 14). In some others, a variable percentage of the total number, a second short anteropetal reaction gradient appears in the posterior region (Fig. 15). In still others the reaction is anteropetal from the beginning (Fig. 16). In some, only the posterior entoplasm, or only the food vacuoles in it, may show reaction; and reaction may be entirely absent in some. This variability as regards reaction in a single experimental lot is greater in *Spirostomum* than in *Paramecium* and *Frontonia*.

As regards the short anteropetal gradient that appears in the posterior region, it is suggested that it is associated with the very frequent backward locomotion, even in culture water and more frequently after addition of reagents. The contractile activity of the large contractile vacuole in this region may perhaps also

play a part in determining this gradient. With high, irritating concentrations of reagents the animals tend to move backward continuously; and the anteropetal gradient appears in all and may extend over the whole length of the body, as in backward-moving *Paramecium*.

With standard or near-standard concentrations the posteropetal reaction gradient is the characteristic reaction pattern over all or most of the body length, with the short anteropetal gradient appearing posteriorly in variable frequency. However, even after appearance of the posteropetal gradient, posteropetal decoloration may follow in 15–30 minutes without oxygen decrease and may proceed until only food vacuoles in the posterior entoplasm show color. With decreasing concentrations absence of reaction anteriorly becomes increasingly frequent with patterns like Figure 16 or with reaction only in posterior entoplasm, only in food vacuoles, or no reaction. Posteropetal decoloration or limitation of reaction to posterior regions and absence of reaction in some individuals in the same experimental lot with others which show reaction are evidently not due to inhibition of reaction by toxic effects, for they appear more frequently as concentrations of reagents decrease and the individuals concerned remain normal and active longer than those showing more reaction. Also, with addition of reagents to increase the concentration the posteropetal reaction pattern appears. Since long-continued aeration does not bring about recoloration in these animals, they probably destroy, rather than reduce, indophenol.

Exposure to either of the reagents in concentrations two to four times standard for periods up to an hour before addition of the other reagent in the same concentrations results in posteropetal retardation or inhibition of reaction, so that whatever reaction does occur is anteropetal. With aniline first, the retardation is greater, extends farther posterior-



FIGS. 14–18.—*Spirostomum:* Fig. 14, completely posteropetal indophenol reaction gradient; also, rapid oxidation of reduced indophenol and dyes. Fig. 15, indophenol reaction gradient and rapid oxidation of reduced indophenol and dyes with posteropetal gradient over most of length, and short anteropetal gradient in posterior region. Fig. 16, slow or partial anteropetal oxidation of reduced indophenol and dyes. Fig. 17, completely posteropetal reduction of indophenol and dyes. Fig. 18, reduction of indophenol and dyes with posteropetal gradient over most of length and short anteropetal gradient in posterior region.

ly, and the animals disintegrate while the reaction is still slight. With α-naphthol first, there is less retardation; the anteropetal reaction usually progresses to the anterior end and becomes deep. With

addition of both agents at the same time in the same concentrations as above, the reaction is posteropetal in almost all individuals, except for the short anteropetal reaction in the posterior region. Evidently, prolonged exposure to either reagent, in concentrations which give rapid reaction when both are added at the same time, retards or inhibits the reaction posteropetally.

With decrease of oxygen following reaction, reduction is posteropetal except for a short anteropetal gradient posteriorly in some individuals (Figs. 17 and 18). The animals usually cytolyze before the reaction progresses so far at the anterior end that reduction is retarded there. Oxidative recoloration, even with long-continued aeration, usually does not attain the original depth of color. In general, the longer the animals remain decolorized, the less the recoloration; apparently, gradual destruction of indophenol occurs during the reduction period. With hydrosulphite, reduction occurs in a few seconds; and if reoxidation occurs immediately after reduction the original color may be almost or quite regained, but if reoxidation is delayed even a few minutes there is much less recoloration, as in *Paramecium*.

In *Spirostomum* the gradient patterns are most conspicuous in the entoplasm, and there seems to be no question as to the reality of the gradient there. This form is more capable of decolorizing indophenol without oxygen decrease than *Paramecium* and *Frontonia*, and many individuals show little or no reaction in concentrations in which *Paramecium* and *Frontonia* react rapidly. The presence of the short anteropetal reaction gradient in a variable percentage of individuals is of special interest in view of the frequent and often continued reversal in direction of locomotion in *Spirostomum*.

REDUCTION AND REOXIDATION OF OTHER INDICATORS

After staining by any of the four dyes used, reduction and reoxidation gradient patterns in both ectoplasm and entoplasm of *Paramecium* and *Frontonia* are similar, as regards direction of progress, to the indophenol patterns. Figures 10, 11, and 13 will serve to indicate reduction; Figures 1, 2, 3, 6, and 12, rapid reoxidation; and Figures 4, 5, and 7, gradual reoxidation in dye concentrations high enough to stain completely but not rapidly toxic. Except with neutral red, reduction and reoxidation patterns of dyes in *Spirostomum* are similar to indophenol patterns (Figs. 14–18). *Spirostomum* is almost completely refractory to staining with neutral red until cytolysis begins or is about to begin. In very high concentrations of oxidized dye or after an hour or more in reduced dye some animals appear to be slightly stained, but they cytolyze almost immediately after staining. In concentrations used for *Paramecium* and *Frontonia*, food vacuoles of *Spirostomum* become deeply stained, oxidized dye accumulates in the fluid of the contractile vacuole, and in the higher of these concentrations the macronucleus may stain while the animals are still active. Also, in spite of the absence of general stain, neutral red is toxic to *Spirostomum*, and cytolysis shows the same gradient patterns as oxidation-reduction.

With reduction of Janus green to red, the oral groove of *Paramecium* and *Spirostomum*, or its margin, and the region about the mouth in *Frontonia* seem to reduce very slightly more rapidly than the general ectoplasm of corresponding body-levels. With the less sharply defined reduction to colorless of the other three dyes, any such difference in reduction becomes uncertain, though it has seemed to be visible in some cases.

When any of the three forms, except *Spirostomum* with neutral red, are sealed in considerable numbers or even brought under a cover glass in small volume of oxidized dye solution immediately after the dye is added, partial posteropetal or even complete reduction may occur without any preceding staining of the reduced part or whole, producing patterns like Figures 4, 5, and 16. The degree of reduction depends, of course, on dye concentration, number of animals, and volume of solution. That this is wholly or largely reduction, not destruction of dye, is shown by the red color of Janus green and by the almost immediate appearance of color on increase of oxygen. With sufficient decrease of oxygen Janus green may be reduced from red to colorless by the animals and reoxidized to red, and, under certain conditions, completely reoxidized to green (p. 265).

Paramecium affords particularly striking examples of the difference between rapid and slow reoxidation, often in a single individual. After reduction by hydrosulphite in open solution the lower levels of the 1-ml. solution on a large depression slide may remain reduced for 5–10 minutes if the liquid is not agitated. Oxygen diffuses in from the surface, and the increase in concentration is very gradual in the deeper parts of the solution. The paramecia are more or less trapped in the less oxidized region; if an individual swims into regions of sufficiently high oxygen content to oxidize the intracellular dye rapidly, oxidation is posteropetal (Figs. 1–3 and 6), and in high concentrations the oxidized dye is rapidly toxic or lethal; movement stops and cytolysis begins almost at once. In lower concentrations as soon as reoxidation begins anteriorly the avoiding reaction occurs; the individual may get back into the reduced region and reduce again posteropetally. With the gradual oxygen increase in the lower levels of solution all individuals show gradual anteropetal reoxidation (Figs. 4, 5, and 7), beginning in the posterior entoplasm. With methylene blue, which is not very toxic, both reoxidations have been observed in the same individual, sometimes more than once, merely in consequence of its entrance into regions of different oxygen content in the solution.

Similar, but less striking effects of rapid and slow reoxidation have been observed in *Frontonia*. *Spirostomum* usually remains at the bottom of the solution, and in absence of aeration its reoxidation is almost entirely or entirely anteropetal. With rapid oxygen increase by aeration, reoxidation becomes more or less completely posteropetal. In both cases the short gradient at the posterior end may or may not be evident.

The direction of progress of reoxidation in the three forms is not dependent on the direction of locomotion. A paramecium revolving on the long axis without advance shows the posteropetal reoxidation gradient as clearly as any other individual when it is pushed by a needle into a region of high oxygen content. Moreover, the swimming animal is often on the way out of the high oxygen before reoxidation is visible in it. *Frontonia* and *Spirostomum* may also show posteropetal reoxidation when not advancing. And, finally, the animals in regions of low oxygen reoxidize anteropetally although they are swimming forward almost or quite continuously. Animals in a drop of reduced solution which is rapidly spread thin on a slide and exposed to air reoxidize posteropetally, although any oxygen gradient present must be at right angles to direction of their movement. These patterns of reduction and reoxidation may, of course, be altered by preceding toxic effects in staining. For example, if the anterior region of *Paramecium* is in-

jured by the oxidized dye in staining, reduction is retarded there, and it reoxidizes first with gradual oxygen increase.

PRIMARY OXIDATION PATTERNS WITH LEUCOBASES

For these primary oxidations 1 ml. of dye solution, in the range of concentrations used for staining without rapid toxic effect, is reduced by hydrosulphite, care being taken to avoid excess of the reducing agent. On introduction into the reduced solution of animals in a drop of culture water the leucobase evidently penetrates almost immediately; and, under properly controlled conditions, intracellular oxidation begins in a few seconds to a minute, except in *Spirostomum* with neutral red, without a trace of oxidation in the solution. This oxidation-gradient pattern is posteropetal like the indophenol reaction pattern (Figs. 1–3, 6, 12, 14), except in the posterior region of some *Spirostomum* individuals (Fig. 15); and, like the indophenol pattern, it is evidently dependent on catalysis by oxidase. The reduced dyes penetrate so rapidly that the posteropetal oxidation gradient appears, even in dye concentrations so low that the solution shows little color before reduction. With the higher concentrations oxidation may be complete over the whole length of the cell in a few seconds, and toxic effects and cytolysis may follow in a few seconds more. With this procedure the gradient pattern is more brilliant and striking than with any other used, because the intracellular leucobase concentration becomes high very rapidly; and when oxidation begins, the color is deep at once (Fig. 19), except in *Spirostomum* with neutral red. In reduced neutral red in the usual range of concentrations the food vacuoles and contents of the contractile vacuole of *Spirostomum* stain almost at once; but general intracellular oxidation of the leucobase becomes visible only after toxic effect and at the beginning, or just before the beginning, of cytolysis. When it does appear, the oxidative gradient pattern is the same as with other dyes.

If any considerable excess of hydrosulphite is used in reducing the dyes, the posteropetal oxidation gradients are less distinct or do not appear at all. Under these conditions oxidation is much less rapid; appears only, or first, in the food vacuoles and posterior entoplasm; and may be entirely anteropetal or may not occur at all except in individuals which swim into regions near the surface of the solution or may occur only very gradually as oxygen diffuses into the solution. It seems evident that appearance of the posteropetal oxidation gradient in completely reduced solutions depends, not merely on oxidase activity, but on presence of oxygen in the animals. With excess of hydrosulphite, oxygen content of the solution becomes so low that intracellular oxygen is used by the animals or diffuses out, and oxidizes hydrosulphite so rapidly that intracellular oxidation of dye can occur only in regions of lower oxygen use, or not at all, except as individuals may swim into regions near the surface. With gradual oxygen increase by diffusion, primary oxidation is like gradual reoxidation after reduction; but with aeration the brilliant posteropetal oxidation patterns may appear, even after large excess of hydrosulphite. The difference in rate of staining by oxidized dye and by oxidation of leucobase is rather striking. With methylene blue, for example, a concentration requiring half an hour for the deep staining of *Paramecium* will stain fully as deeply in 1–2 minutes by intracellular oxidation of the reduced dye. Differences in rate of staining with oxidized and reduced neutral red and Nile blue sulphate are somewhat

less but still considerable. With Janus green the difference in rate of staining by the red, partially reduced form and the oxidized dye is perhaps even greater than with methylene blue.

Intracellular oxidation of reduced Janus green is of particular interest. The completely reduced, colorless form undergoes rapid intracellular oxidation to brilliant red. This red, partially reduced form is said to be irreversible as regards reoxidation to green. This appears to be true *in vitro*, but intracellular reoxidation to green is possible in all three ciliates. For this purpose animals not previously exposed to the dye are brought, in a drop or two of culture water, into concentrations of partially reduced red dye which have been found not to be rapidly toxic. The red form is easily obtained by reducing oxidized Janus green with hydrosulphite; if it goes beyond the red to the colorless stage, a little aeration brings it back to red, and the red form is stable in water. Under these conditions gradual intracellular oxidation to green occurs posteropetally without any red stain in the animals, except that in some *Spirostomum* individuals the short anteropetal gradient may become visible at the posterior end. With Janus green 1/200,000 reduced to colorless, oxidation to green occurs with little or no evidence of the red stage. That intracellular oxidized dye is actually present in these cases is evident from the greenish color of the animals and can be further demonstrated by intracellular reduction to red with a little hydrosulphite. In animals which have already oxidized the fully reduced colorless dye to red, further oxidation to green may still occur to some extent in low, not rapidly toxic concentrations.

The difference in toxicity of oxidized and reduced dyes is striking. Animals may live, apparently quite uninjured, until affected by lack of oxygen in concentrations of leucobase-hydrosulphite which become toxic in a minute or two on oxidation. Exposure for 1 minute to such concentrations is sufficient for attainment of intracellular concentrations which very rapidly become toxic on oxidation.

With very high concentrations of the dyes reduced by hydrosulphite there is immediate irritation when oxidation begins. As in high concentrations of indophenol reagents, *Paramecium* moves rapidly and continuously, or almost continuously, backward, either without visible oxidation anteriorly or with a trace of color at the anterior tip of the cell. Under these conditions oxidation becomes visible at the posterior end almost at once, as in Figure 8; coloration becomes deep there and progresses anteropetally over the whole length of the cell in a few seconds, and cytolysis soon follows in the same direction. With somewhat lower concentrations and less extreme irritation the posteropetal oxidation gradient may become distinct and extend some distance posteriorly, the two gradients usually meeting some-

FIG. 19.—*Paramecium:* early stage of rapid primary oxidation of dye from leucobase solutions with deep staining anteriorly before color is visible elsewhere.

what anterior to the middle, as in Figure 9. If hydrosulphite is added in amount sufficient to prevent intracellular oxidation entirely, there is little or no irritation or backward movement, even in dye concentrations that are highly irri-

FIG. 20.—*Spirostomum:* rapid primary oxidation in high concentrations of dye leucobases with continuous backward locomotion; the anteropetal gradient appears first and extends over most of the length, the short posteropetal gradient appearing later.

tating on oxidation; but, as soon as oxygen is introduced into these solutions by slight aeration, irritation and rapid backward movement appear at once and intracellular oxidation is entirely, or almost entirely, anteropetal. These data indicate that the irritation and reversal of locomotion result from the earliest stages of oxidation at the anterior tip of the cell, even though color is not visible there. Appearance anteriorly of the posteropetal oxidation gradient in animals moving rapidly backward when irritation is not extreme shows that this gradient is not determined by direction of locomotion. Here, as with indophenol, the anteropetal oxidation gradient in the backward-moving animals suggests an increase posteriorly in oxidase activity sufficient to reverse more or less completely the normal posteropetal gradient. These observations confirm and extend the earlier data on reversal of the oxidation gradient in *Paramecium caudatum* in methylene blue leucobase plus reducing agent (Child, 1934).

In *Frontonia* reversal of locomotion is less extreme in character and less continuous, as with indophenol reagents; but anteropetal oxidation does occur in the posterior region and may progress over the whole length, though the posteropetal gradient often appears anteriorly. In *Spirostomum* backward locomotion and anteropetal oxidation over more or less of the body-length occur in the high leucobase-hydrosulphite concentrations, but the posteropetal gradient usually also appears slightly later (Fig. 20). The body-level at which the two meet becomes more anterior, i.e., reversal becomes more complete, with increasing concentrations.

In leucobase concentrations not rapidly injurious, intracellular oxidation may be followed by posteropetal decoloration within 2–3 minutes in the deeper parts of open solutions, where there is still little oxygen, or in solutions under cover glass or sealed, which remain reduced. In these cases the primary oxidation of the dye by oxidase and intracellular oxygen is followed by reduction in consequence of low oxygen content of the solution. Intracellular oxygen is no longer available. After this second-

ary reduction, reoxidation is possible only as the oxygen content of the solution is increased.

PATTERNS OF STAINING, THEIR MODIFICATIONS, AND THE QUESTION OF INTRACELLULAR DESTRUCTION OF INDICATORS

Mention of the patterns of staining with oxidized dyes has been delayed to this time because they are best considered in the light of the data on reduction and oxidation. A posteropetal staining gradient was observed in *Paramecium* and *Dileptus* by MacArthur (1921) and again in *Paramecium* by Child and Deviney (1926) and by Child (1934). With rapidly staining concentrations of the four dyes used, a posteropetal staining gradient appears anteriorly almost immediately, except in *Spirostomum* with neutral red. This is primarily ectoplasmic in *Paramecium* and *Frontonia* and apparently both ectoplasmic and entoplasmic in *Spirostomum*. With neutral red the gradient is very slight and soon disappears with the progress of general staining. With the other dyes the differential is greater, and a staining gradient may be continuously visible until the anterior region becomes deeply colored as the dye becomes differentially toxic. With lower dye concentrations the staining gradient may soon become indistinguishable as general staining progresses. In later stages the anterior end of *Paramecium* becomes very deep in color, except with neutral red, and may shrink laterally as cytolysis begins. With neutral red more or less change of color toward alkalinity is usually evident with toxic effect, though with very high concentrations the anterior end may become more deeply colored and shrink. *Frontonia* may also show a slight change in color of neutral red toward alkalinity anteriorly. In both forms it may represent early toxic effect, although it occurs while the animals are still normal in form and behavior. Later, during cytolysis, the whole body of the paramecium becomes yellow posteropetally. With the higher concentrations of neutral red, parts of the canal system near the contractile vacuoles stain in a somewhat more acid color than the ectoplasm and so deeply that the canals appear opaque black in the red cell body. The wall of the vacuole is not specially stained. Staining of the canal system has not been observed in *Frontonia* and *Spirostomum*, nor has it been seen in *Paramecium* with other dyes than neutral red. In general, *Spirostomum* stains less rapidly than the other forms with the oxidized dyes Janus green, Nile blue sulphate, and methylene blue and cytolyzes before the color becomes deep.

The entoplasms of *Paramecium* and *Frontonia*, particularly recently formed food vacuoles and other inclusions, stain as rapidly as, or more rapidly than, ectoplasm, except in high dye concentrations. In *Paramecium* the postoral entoplasm usually becomes deeper in color than the preoral. This is not merely an apparent difference due to the greater diameter of the postoral region; there are more food vacuoles and other inclusions which stain in the postoral than in the preoral entoplasm. Moreover, the postoral entoplasm usually stains more rapidly than preoral; with neutral red it may become distinctly stained in a few seconds. In *Frontonia* food vacuoles and other inclusions are often more numerous posteriorly and stain more deeply than anterior regions; otherwise there is little difference. In *Spirostomum* the finely granular entoplasm, usually with only a few food vacuoles in its posterior region, shows a distinct posteropetal staining gradient in earlier stages of staining. It is perhaps of interest to point out that the postero-

petal staining gradient appearing in the ectoplasm of all three forms and also in the entoplasm of *Spirostomum* in earlier stages of staining with rapidly staining dye concentrations is similar to the indophenol reaction gradient with concentrations of reagents near the standard. The indophenol gradient is not due to a differential in permeability to the reagents, and the staining gradient evidently is not the result of a differential permeability to the dyes, since the postoral entoplasm or its inclusions may stain as rapidly as, or even more rapidly than, the anterior ectoplasm and so rapidly that intake by mouth appears to be excluded. Both the staining and the indophenol pattern are expressions of the physiological gradient pattern, and their similarity brings up the problem of the nature of vital staining. This problem was discussed earlier, in relation to staining gradients by MacArthur (1921). Here it need only be noted that the staining gradients suggest a possible relation of some sort between staining by oxidized dyes and oxidase activity. If there is any such relation, it is less evident with neutral red than with the other three dyes used.

Certain modifications of staining pattern appearing with low dye concentrations seem to indicate an irreversible decoloration or destruction of the dyes by the living organisms. This decoloration occurs without oxygen decrease and color does not return with high oxygen. In general these modifications consist in posteropetal decoloration after staining; or in still lower concentrations, anterior regions remain completely unstained and color increases posteropetally in both ectoplasm and entoplasm; or in *Paramecium* the ectoplasm remains without color and only the posterior entoplasm stains. These staining patterns are essentially like those with low concentrations of indophenol reagents (e.g., Figs. 4 and 5), and they appear like stages of posteropetal reduction. In *Frontonia* they show less differential than in *Paramecium;* in *Spirostomum* they are distinct and usually appear with higher dye concentrations than in the other forms. In *Paramecium* these modifications appear, to some extent, in methylene blue and neutral red from about 1/25,000 down; in Nile blue sulphate from 1/100,000 down, and are evident within a few minutes. With Janus green 1/200,000, *Spirostomum* may show primary posteropetal absence of color; in *Paramecium* slight secondary posteropetal decoloration may be evident after an hour or more but is usually terminated by toxic effect, but even in 1/800,000 the staining gradient is usually primarily posteropetal. The red, partially reduced Janus green is apparently decolorized somewhat more readily than the oxidized dye by *Paramecium*. *Frontonia* shows slight posteropetal decoloration with lower dye concentrations than the other forms. The outer, finely granular entoplasmic zone, usually distinguishable from the inner entoplasm, in *Frontonia* stains much less deeply than the inner mass and often seems to show a more distinct decoloration gradient, though it is, of course, seen through the ectoplasm. In animals containing little food the whole entoplasm may show a posteropetal decoloration gradient. Large *Frontonia* individuals with numerous food vacuoles often show, after staining with oxidized Janus green, a shift of color toward red, sometimes to red, in the interior of the inner entoplasmic mass. This is a reduction indicating low oxygen content in the interior of the entoplasm. It may occur without experimental oxygen decrease but is more frequent in aggregations.

Staining in these ciliates appears to be

primarily diffuse without distinct localization, adsorption, or concentration on or in any discrete granules or distinguishable particles, except the food vacuoles and other inclusions in the entoplasm. With high dye concentrations cytolysis may occur while the stain is still diffuse. With lower concentrations staining appears primarily diffuse in ectoplasm and the ground substance of the entoplasm, as distinguished from the inclusions; but in *Paramecium* and *Frontonia* it gradually becomes increasingly granular in appearance, the dyes apparently becoming increasingly localized or concentrated on or in cytoplasmic granules or particles in both ectoplasm and entoplasm. Differential decoloration occurs almost entirely in the diffuse stage of staining, and reduction is retarded or inhibited after the granular stage becomes distinct. With low dye concentrations the ectoplasm may not stain at all or may decolorize before the granular stage is reached, and this appears only in the entoplasm; or ectoplasmic decoloration may occur anteriorly and the granular stain posteriorly. A similar gradual concentration of dye on cytoplasmic granules with retardation of reduction has been observed in stages of echinoderm development (Child, 1936). *Spirostomum* usually disintegrates before it becomes deeply colored by oxidized dyes, and it shows little or no evidence of the granular localization of dye. It stains more rapidly and deeply by the primary intracellular oxidation of leucobases than by oxidized dyes but does not show distinct granular staining, except perhaps in the posterior entoplasm.

A few data concerning modifications of staining pattern in *Spirostomum* with oxidized and reduced Janus green are given in some detail. In lots from a flourishing culture, slight staining occurred in oxidized Janus green 1/100,000 in $2\frac{1}{2}$–3 hours, some individuals having slight posteropetal, others anteropetal, gradients with the anterior half or more unstained. After 4–5 hours most individuals which earlier showed posteropetal staining were undergoing posteropetal decoloration to colorless without any trace of red. In a lot in oxidized Janus green 1/200,000 there was only slight, almost entirely anteropetal, stain after 4–$4\frac{1}{2}$ hours. With this lot the dye was then reduced through red to colorless by hydrosulphite. The animals oxidized to red posteropetally, and posteropetal loss of red color followed gradually in some individuals. With low concentrations of Janus green it is sometimes difficult to determine by direct observation whether the animals are decolorizing the red stage or oxidizing it to green (p. 265), since the intracellular green in low concentrations is difficult to distinguish with certainty. However, this question can usually be decided by another reduction by hydrosulphite. If irreversible decoloration or destruction of dye has occurred, no red color appears in the regions concerned after addition of hydrosulphite; but in case of intracellular oxidation from red to green, the regions involved become red, and the intracellular red of partially reduced Janus green is much more brilliant and more readily seen in very low concentrations than the green.

In the attempt to obtain further evidence concerning entoplasmic decoloration, lots have been kept in low dye concentrations in darkness, except for examinations once or twice daily, either until death or until no further change was evident. In oxidized methylene blue 1/400,000 *Paramecium* ectoplasm became slightly stained anteropetally at first, in most individuals only postorally, but decolorized completely in almost all within 2–3 hours. Entoplasm became

much more deeply stained. After 3 days only scattered entoplasmic granules were stained, mostly in the postoral entoplasm; and in some individuals even these granules were almost colorless. After 5 days some 50 per cent were still alive, and almost or completely colorless. Cytolytic disintegration appeared first in the postoral region. Observations were then concluded. *Frontonia* in the same lots stained much more rapidly and deeply than *Paramecium* and showed very little ectoplasmic and no entoplasmic decoloration, and all individuals died within 2 days. *Spirostomum* did not stain at all or stained very slightly in the posterior entoplasm, and all individuals died within 5 days. Color clearly visible in the solution containing the animals at the beginning gradually disappeared completely, and at the same time intracellular color decreased.

In neutral red 1/200,000, *Paramecium* ectoplasm showed slight posteropetal stain at first but soon became completely colorless. Entoplasm stained much more deeply, and most deeply postorally. Gradual entoplasmic decoloration occurred, but color was still present after 10 days in the interior of the postoral entoplasm. In *Frontonia*, ectoplasm and the clear outer entoplasmic zone showed a slight anteropetal staining gradient, and the inner entoplasm became deeply stained. The ectoplasmic stain gradually became granular anteropetally over the posterior third or half; anteriorly ectoplasm and clear entoplasmic zone gradually became colorless in 2–3 days; and in the inner entoplasm color decreased anteropetally. All cytolyzed within 4 days. In *Spirostomum* only food vacuoles stained. Some individuals gradually shortened within 4 days and later cytolyzed, but some were still normal after 10 days.

In Nile blue sulphate 1/2,000,000, *Paramecium* ectoplasm was not visibly stained at any time; entoplasm stained anteropetally with little or no color anteriorly. In the course of 7 days most individuals became almost colorless, with a few scattered granules in the postoral entoplasm showing faint color; a few showed deeper color in the postoral entoplasm and slight color preorally. After 10 days all were dead. In *Frontonia* ectoplasm and clear entoplasmic zone stained anteropetally, more or less of the anterior region remaining colorless; in most individuals all except the inner entoplasm became colorless within 2 days; in 3–4 days some posteropetal decoloration appeared in the inner entoplasm. After 5 days all were cytolyzed. *Spirostomum* stained only in the posterior entoplasm and food vacuoles; after 6 days they were without visible color and were still normal and colorless after 10 days.

In Janus green 1/800,000, *Paramecium* and *Frontonia* showed at first a trace of posteropetal ectoplasmic staining gradient and after several hours a slight posteropetal decoloration with no trace of red, and all individuals cytolyzed within 24 hours. *Spirostomum* did not stain appreciably, and after reduction by hydrosulphite it showed no red.

The primary anteropetal staining gradient with little or no color anteriorly and the secondary posteropetal decoloration in low concentrations of dyes, as well as of indophenol (p. 268), the complete absence of red in decoloration of Janus green, and the complete ineffectiveness of long-continued aeration or even pure oxygen in increasing color suggest that the oxidized indicators are gradually destroyed rather than reversibly reduced within the cells and that destruction takes place within the entoplasm as well as in the ectoplasm, but more slowly. Moreover, the differential characteristic of decoloration indicates that the de-

struction is also an expression of the physiological gradient pattern.

Another line of experiment with *Paramecium* material provides further evidence concerning intracellular destruction of indicators, both indophenol and dyes. With standard concentrations of indophenol reagents or half or double these, or with any concentrations of the oxidized dyes that will stain readily, paramecia in large numbers are sealed in 0.5 ml. of solution on a depression slide without air bubbles. Under these conditions oxygen decrease is so rapid that little or no indophenol reaction or stain appears; if any does appear at first, it is very soon reduced. Controls in the same concentrations open to air react or stain rapidly with the usual posteropetal gradient and sooner or later die. After 1–2½ or 3 hours, depending on the number of animals present, most of the sealed animals are slow or motionless, evidently because of lack of oxygen, since opening and aeration are followed almost at once by recovery. However, even after an hour or more following opening and aeration, there is only very slight or no indophenol reaction and little visible color of dyes, often only in the postoral entoplasm, while the controls have reacted or stained deeply and are dead or dying. With the dyes color of the solution may also be considerably less than at the time of sealing, indicating continuous uptake and destruction of dye by the animals.

In this line of experiment Janus green is less satisfactory than the other dyes used. During the period of sealing, the animals may stain red with the partially reduced dye and then become colorless posteropetally; but reduction of Janus green to colorless occurs only when oxygen concentration has become so low that effects of lack of oxygen soon follow. On opening and aerating before these effects become irreversible the animals become red and usually appear somewhat less deeply red than in the earlier red stage and often with posteropetal increase of color. If a toxic effect of the dye does not prevent, they may gradually reoxidize to green, also with posteropetal increase in depth of color. Apparently there is some intracellular destruction of Janus green, but with the oxidized dye it is less rapid and less complete than with the other dyes.

With moderate indophenol reaction preceding reduction, color is usually less deep after reduction and reoxidation than in controls in the same concentrations of reagents for the same period but without reduction. Also, the reoxidation gradient may be anteropetal even with very rapid oxygen increase. Apparently more or less posteropetal destruction of intracellular indophenol has occurred during reduction. The experiments involving reduced indophenol indicate that it is destroyed more rapidly than the oxidized form.

With removal of oxygen by hydrosulphite, reduction of intracellular indophenol occurs as in sealed preparations, but much more rapidly. The solution becomes acid on addition of hydrosulphite, but after reoxidation and thorough aeration is again alkaline. If the reduced solution is aerated as soon as reduction is complete, or complete except in postoral entoplasm, that is, within a minute or two after addition of hydrosulphite, there is more or less intracellular reoxidation, though often not to the depth of color preceding reduction. Moreover, these animals gradually decolorize posteropetally again. When hydrosulphite is added to solutions of indophenol reagents in concentrations used for the reaction, with addition of *Paramecium* 10–15 minutes later, after thorough aera-

tion, there is no reaction. In open preparations solutions of the reagents gradually become bluish or purplish in consequence of indophenol formation and oxidation of aniline *in vitro*. After the addition of hydrosulphite they remain colorless, even though thoroughly aerated. These data on the use of hydrosulphite for reduction of intracellular indophenol indicate that this reagent, or the products of its oxidation, can destroy indophenol within the *Paramecium* cell and in solution and also one or both of the indophenol reagents. They are given in detail because it is a point of some importance in the use of hydrosulphite with indophenol that reoxidation after reduction is incomplete or absent. With the dyes used, no such effect of hydrosulphite is evident.

Gradual decoloration also follows extreme dilution of the solution after indophenol reaction or staining, provided toxic effects have not occurred. It is possible that in these cases the intracellular indicator gradually diffuses out, though there is no evidence of loss in that way; and in view of other lines of evidence, gradual destruction is probable.

Attempts have been made, with *Paramecium* as material, to determine whether destruction of the indophenol reagents occurs. After long exposures up to 24 hours in closed containers to low aniline concentrations, not appreciably toxic during the exposure period, there is much less rapid reaction on addition of α-naphthol than in controls with both reagents added at the same time in the same concentrations. This difference, however, is not proof of destruction of aniline by *Paramecium*. The same low concentrations of aniline kept in closed containers without *Paramecium* for the same periods give much less reaction after addition of *Paramecium* and α-naphthol than controls. In these low concentrations the aniline gradually undergoes oxidative and perhaps other alteration. Similar exposures of *Paramecium* to α-naphthol before addition of aniline give uncertain results. There is apparently slightly less rapid reaction than in controls, but the difference does not indicate any considerable destruction of α-naphthol by the animals.

Whether or to what extent the refractory character of *Spirostomum* as regards neutral red involves destruction of the dye has not been determined. The dye apparently enters rapidly, since, with the higher concentrations used, it becomes visible in the fluid of the contractile vacuole at the posterior end of the body in a few seconds and since food vacuoles just anterior to the contractile vacuole stain almost or quite as rapidly. It seems improbable that intake through the mouth can account for this rapid appearance of color in contractile and food vacuoles almost half the body-length distant from the mouth. In high concentrations the macronucleus may also stain while the animals are still moving but are somewhat shortened. Without general staining neutral red is toxic to *Spirostomum*. In the higher concentrations of oxidized dye cytolysis begins with shortening and distortion, progressing posteropetally from the anterior end, often with a second anteropetal cytolysis at the posterior end. The cytolyzing regions stain at once and become deeply colored. In a lower range of concentrations of oxidized dye the animals gradually shorten and become almost or quite spherical while still capable of slow movement and without any general stain. Reduced neutral red is little more effective than oxidized in staining. Contents of the contractile vacuole and food vacuoles oxidize the reduced dye, and later the macronucleus stains; after an hour or more in reduced neutral red some individuals show slight

general stain, but in these cytolysis and deeper stain follow almost at once. *Spirostomum* apparently either reduces neutral red in the cytoplasm, even with high oxygen content of the solution, or destroys it, or both, until toxic effect occurs. This ciliate is apparently, or can be, almost or quite anaerobic. It remains alive and normally active for periods up to 2 or 3 days in 0.5 ml. of culture water deprived of oxygen by hydrosulphite and sealed without air bubbles. Under these conditions *Paramecium* and *Frontonia* live, at most, only a few hours.

It has been found by Barron and his co-workers that methylene blue and certain other vital dyes increase oxygen uptake of mammalian erythrocytes and unfertilized eggs of the sea urchin, starfish, and *Nereis*.[3] The increase by methylene blue is 80–100 per cent in sea-urchin eggs, 200–250 per cent in starfish eggs. Janus green was found to increase oxygen uptake only slightly (10 per cent), and neutral red produced no increase. If there is any similar increase in oxygen uptake by the dyes in the ciliate material of the present paper, intracellular reduction in higher oxygen concentrations than before the increase will become possible. The gradual posteropetal decoloration after primary staining of *Paramecium* and *Spirostomum* in moderate concentrations of the oxidized indicators and the partial or complete absence of ectoplasmic stain in low concentrations suggest that the animals may have become able to reduce the dyes without oxygen decrease. However, long-continued aeration or bubbling pure oxygen through the solutions produces no recoloration in many cases. If the decoloration or lack of stain is due entirely to reduction, the animals keep the dyes reduced even in solutions saturated with oxygen. In other experimental lots some recoloration or staining follows oxygen increase, showing that increased reducing power is concerned in the decoloration or lack of stain in those cases. In view of all the evidence, the conclusion appears justified that gradual destruction, as well as increased reduction, may play a part in the irreversible secondary decolorations and the partial failures to react or stain in low concentrations. The evidence also suggests that the catalyzing effect of the indicators in the lower concentrations may increase the ability of the cells to destroy them.

Barron and Hoffman (1930) found that neutral red produced no increase in oxygen uptake. In *Paramecium*, however, decoloration or absence of stain, partially reversible by high oxygen or irreversible, appears with neutral red, as with methylene blue, but with lower absolute concentrations because neutral red is more toxic than methylene blue. The concentration of neutral red used by Barron and Hoffman is higher than those showing increased reduction or destruction in *Paramecium*. Increase of oxygen uptake might have appeared in their material with lower concentrations of neutral red. Whether *Spirostomum* reduces or destroys neutral red remains uncertain. In the slow and slight decoloration of Janus green the complete absence of the red stage characteristic of reduction suggests destruction of the dye rather than reduction.

The point of greatest interest as regards the modifications of gradient pattern under special experimental conditions with the indicators is that they are referable to, and expressions of, an oxidation-reduction gradient pattern which is evidently a fundamental feature of the organization of these ciliates. It was pointed out earlier in this section (p.

[3] Harrop and Barron, 1928; Barron and Harrop, 1928; Barron, 1929; Barron and Hoffman, 1930; Barron and Hamburger, 1932.

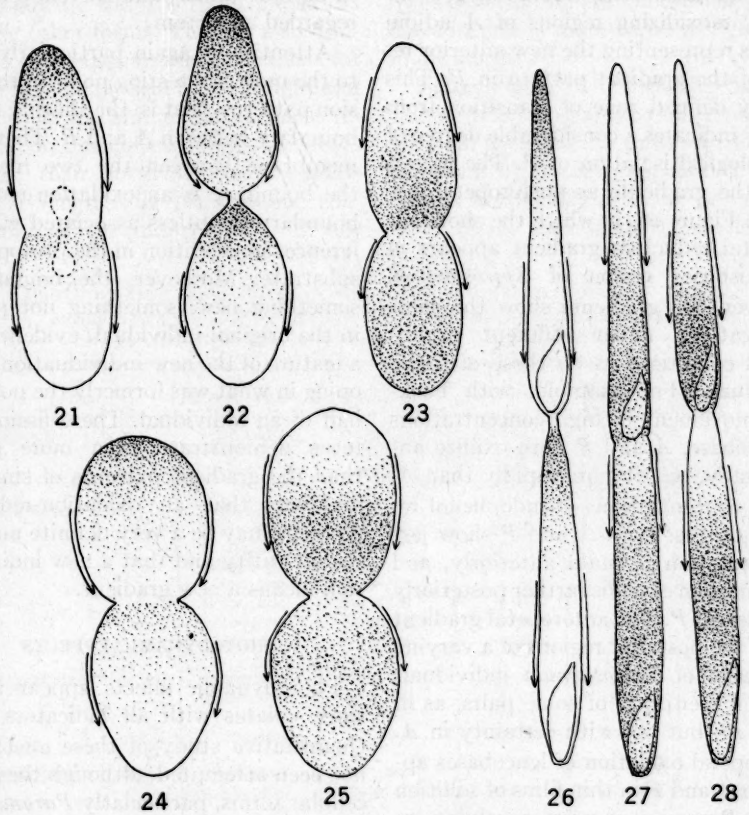
267) that the similarity of patterns of staining and of indophenol reaction raise the question of the nature of vital staining. It is now evident that the oxidation-reduction patterns in general and their modifications under experimental conditions involve this question.

In this connection brief mention is made here of a few experiments on staining in relation to temperature. These consisted of lots containing all three ciliates brought into concentrations of methylene blue high enough to avoid decoloration and kept at temperatures of 8°–10° C. and 20°–23° C. in darkness except for brief periodic examinations. These lots consistently gave temperature coefficients not varying greatly from 2 for certain stages of staining, which could be fairly well compared, and for cytolysis by the dye in all three species, suggesting that chemical factors may be involved. That the few experiments performed have more than merely suggestive significance is, of course, not assumed.

OXIDATION-REDUCTION PATTERNS IN FISSION

In order to avoid frequent and confusing repetitions of "anterior" and "posterior," the anterior member of a fission pair is designated by *A*, the posterior member by *P*. Until toxic effects of the indicators appear, fission stages of the three ciliates show, in general, more rapid indophenol reaction, more rapid oxidation of reduced indophenol and dyes, except with excess of hydrosulphite, and more rapid reduction of indophenol and oxidized dyes in *A* than in *P*. Figures 21 and 22 indicate indophenol reaction in an early and a late stage of fission in *Paramecium;* reoxidation after staining with oxidized dyes followed by reduction and after reduction of indophenol shows similar stages when oxygen increase is rapid; with gradual oxygen increase reoxidation is more rapid in *P* than in *A*. Reduction in a fission pair of *Paramecium* is indicated in Figure 23; *A* reduces more rapidly than *P*. A stage of indophenol reaction in a fission pair of *Frontonia* is indicated in Figure 24; rapid reoxidation after reduction of indophenol or dyes is essentially similar; *A* reacts and reoxidizes more rapidly than *P*. Figure 25 indicates reduction in a *Frontonia* pair; *A* reduces more rapidly than *P*. A stage of indophenol reaction in advanced fission of *Spirostomum* is indicated in Figure 26. At this late stage of fission *A* still reacts slightly more rapidly than *P*. Rapid reoxidation after reduction is essentially similar. Figure 27 indicates much more rapid reduction in *A* than in *P* in an early fission stage. In Figure 28, also indicating a stage of reduction, more rapid reduction is indicated in *A*. Even after separation *A* and *P* are distinguishable in *Paramecium* and *Frontonia* by the difference in rate of reaction, reduction, and reoxidation. Differences in the two members after separation seem to be somewhat less in *Spirostomum* but have apparently been visible in some cases. These observations indicate persistence during and after fission of a real physiological difference in *A* and *P* similar to the difference in anterior and posterior regions of a single individual. They are in line with the earlier observations of Petersen (1927) and Hinrichs (1928), showing that in *Paramecium* anterior products of fission tend to divide more rapidly than posterior products, at least for a time.

Reaction, primary leucobase oxidation, and reoxidation gradients, and also reduction gradients, are distinct in *A* and *P* of the pairs, as indicated in Figures 21–28. This is particularly conspicuous on the two sides of the fission zone where the less rapidly reacting, oxidizing,



FIGS. 21–28.—Oxidation-reduction patterns in fission. Figs. 21–23, *Paramecium:* Fig. 21, early fission with early stage of posteropetal indophenol reaction or rapid oxidation of reduced indophenol or dyes; the transition at the fission zone from anterior to posterior member is sharply defined; reaction and oxidation are more rapid in the anterior than in the posterior member. Fig. 22, late fission with posteropetal indophenol reaction or rapid oxidation of reduced indophenol or dyes; anterior member still in advance of posterior member, but the two anterior regions differ less than in Fig. 21. Fig. 23, posteropetal reduction of indophenol and dyes, more rapid in anterior than in posterior member. Figs. 24 and 25. *Frontonia:* Fig. 24, posteropetal indophenol reaction and rapid oxidation of reduced indophenol and dyes, more rapid in anterior than in posterior member. Fig. 25, posteropetal reduction of indophenol and dyes, more rapid in anterior than in posterior member. Figs. 26–28, *Spirostomum:* Fig. 26, early stage of posteropetal indophenol reaction or rapid oxidation of reduced indophenol or dyes; anterior member slightly in advance of posterior. Fig. 27, posteropetal reduction of indophenol and dyes in early fission; anterior member in advance of posterior. Fig. 28, later stage of reduction in more advanced fission; anterior member in advance of posterior; very slight anteropetal reduction in posterior region of posterior member.

reducing, and, with rapid oxygen increase, reoxidizing regions of *A* adjoin regions representing the new anterior region of the gradient pattern in *P*. This sharply defined zone of transition from *A* to *P* indicates a considerable degree of physiological isolation of *P*. The figures show the gradients as posteropetal, except in Figure 28, in which the short anteropetal reduction gradient appears in the posterior region of *Spirostomum*. However, the gradients show the same modifications under different experimental conditions as do those of single individuals. For example, with backward movement in high concentrations of leucobase, *A* and *P* both oxidize anteropetally, but *P* more rapidly than *A*. In low concentrations of indophenol reagents or dyes both *A* and *P* show less or no reaction or stain anteriorly, and this condition extends farther posteriorly in *A* than in *P*. The anteropetal gradient seen in the posterior regions of a varying percentage of *Spirostomum* individuals has been seen in *P* of some pairs, as in Figure 28, but not with certainty in *A*. Posteropetal oxidation of leucobases appears in *A* and *P* in thin films of solution and in *Paramecium* pairs revolving on the long axis without advance when they are pushed into regions of high oxygen content. In short, in so far as observed, *A* and *P* are distinct individuals, as far as gradient patterns are concerned, from early stages of fission; but *P*, at least in *Paramecium*, is still somewhat "more posterior" than *A*, even after separation. Both *A* and *P* are undergoing reconstitution from halves to wholes, but *P* does not attain the physiological levels of *A* at the time of separation. In some cases reduction of Janus green to red is perhaps very slightly more rapid in the reorganizing oral groove; but it has not been possible to observe this consistently in all individuals, and therefore it is not regarded as certain.

Attention is again particularly called to the most interesting point in these fission patterns, that is, the sharply defined boundary between *A* and *P*. There is no membrane between the two members; the boundary is an oxidation-reduction boundary doubtless associated with differences in condition in the protoplasmic substrate. Moreover, the boundary is something new, something not present in the original individual; evidently it is a feature of the new individuation developing in what was formerly the posterior half of an individual. These fission patterns demonstrate even more clearly than the gradient patterns of single individuals that an oxidation-reduction gradient may be a very definite and persistent entity and that a new individuation means a new gradient.

PHOTODYNAMIC EFFECTS

Photodynamic effects appear in the three ciliates with all indicators used. Quantitative study of these effects has not been attempted, although these unicellular forms, particularly *Paramecium*, appear to be favorable material. Certain features of the photodynamic effects are of interest in relation to oxidation-reduction and to gradient pattern. Specific differences in rate of indophenol reaction and staining and in photodynamic sensitivity render comparative data of little significance. For example, *Spirostomum* is photodynamically sensitive to neutral red without appreciable staining and is killed in sunlight almost or quite without visible indophenol reaction or staining in the other indicators, while *Frontonia* and *Paramecium* usually show at least slight reaction or stain before any photodynamic effect appears. Moreover, *Spirostomum* is much more sensitive to sun-

light in culture water without indicators than the other forms. There is also difficulty in providing an adequate basis for comparison of survival times in light and darkness. If the animals in darkness remain in solutions of indophenol reagents or dyes, their survival time is determined by increasing intracellular concentration of the indicators; or in low concentrations they react or stain only partially or undergo differential decoloration. If they are returned to culture water without indicators, there is usually more or less decoloration, in consequence of destruction or loss of intracellular indicators. Even if the two lots in light and darkness are kept in equal small volumes of solution, so that only a certain degree of reaction or staining is possible, survival time in darkness may be considerably prolonged by destruction or reduction, while in light decoloration may be largely or entirely prevented by early lethal effects. On the other hand, decoloration is more rapid in light than in darkness.

Table 1 gives a few of the data obtained indicating approximate ranges of survival times for the three species as determined by earliest appearance of cytolysis and cytolysis in 95 per cent or more of the individuals in the lot. Not infrequently a few individuals, sometimes a single one, will live much longer than the rest. These are not considered in recording the final survival times. In all cases the lots were exposed to full sunlight immediately after addition of the oxidized dyes or after 15 minutes' exposure to indophenol reagents. All lots were in approximately 1 ml. of solution on large depression slides without cover. Under the heading "Conditions" the word "glass" means sunlight through glass of a window; "no glass," direct exposure without intervening glass. The

TABLE 1

PHOTODYNAMIC SENSITIVITY: RANGES OF SURVIVAL TIMES IN MINUTES IN FULL SUNLIGHT 15 MINUTES AFTER ADDITION OF INDOPHENOL REAGENTS AND IMMEDIATELY AFTER ADDITION OF OXIDIZED DYES

Indicator	Concentrations	Conditions	*Paramecium*	*Frontonia*	*Spirostomum*
Indophenol	Standard×2	Stage after 15 minutes' reaction	18–45	8–25	23–45
Methylene blue	1/200,000	No glass	2–7	4–7	3–5
	1/100,000	Heat filter	1–16	2–15	2–7
	1/100,000	Glass	3–10	5–10	5–8
	1/100,000	Glass	4–9	2–9	3–6
	1/100,000	No glass	2–9	2–9	1–5
	1/100,000	No glass	3–8	3–6	4–6
	1/100,000	No glass	2–7	4–7	3–5
	1/100,000	No glass	1–15	2–15	2–7
Janus green	1/200,000	Heat filter	3–25	15–40	15–22
	1/200,000	No glass	3–25	15–37	10–15
	1/200,000	No glass	20–35	27–45	30–45
	1/200,000	No glass	17–35	20–35	25–40
Nile blue sulphate	1/500,000	No glass	7–25	15–25	25–38
	1/500,000	Stage	6–26	12–26	22–31
Neutral red	1/200,000	Stage	2–13	6–16	2–7
	1/100,000	Stage	2–16	4–15	2–6
	1/100,000	Stage	1–10	1–12	1–5

"heat filter" used in some cases was 3 inches of cold water in a flat-bottomed glass container; in these cases the sunlight passed through both water and glass of the filter but not through window glass. All lots were exposed with white paper under the slide, except a few kept continuously on the microscope stage at open window, as indicated in the table by the word "stage," under "Conditions." All lots tabulated were run on cool days, and temperature changes on the slides were slight. The last two lots in Janus green were run at considerably lower temperature than the others and show longer survival times, particularly as regards beginning of cytolysis.

The minimum and maximum survival times given in the table represent approximately the limits of a frequency curve, the frequency of cytolysis increasing from perhaps one or a very few individuals at the beginning to a maximum and decreasing again until only a very few, perhaps only one, may remain unaffected. The tabulated lots were taken from apparently vigorous, reproducing cultures. Some evidence obtained indicates that photodynamic sensitivity varies with nutritive and perhaps with other cultural conditions.

The table indicates that the maximum survival time of *Spirostomum* in methylene blue is, in general, somewhat less than those of *Frontonia* and *Paramecium*, which differ very little. In Janus green *Frontonia* and *Spirostomum* appear slightly less sensitive than *Paramecium*. In Nile blue sulphate some *Spirostomum* individuals live longer, and in neutral red all cytolyze earlier, though without cytoplasmic stain preceding cytolysis, than *Paramecium* and *Frontonia*. These appear to be specific differences, but nothing is known concerning their physiological basis. There is little difference between 1/200,000 and 1/100,000 methylene blue and neutral red; and between lots with heat filter, or with light through glass, and those with no glass. The second and the last methylene blue lots from the same culture show a wider range of survival times in *Paramecium* and *Frontonia* than other lots, but the same for *Frontonia* and *Spirostomum* and almost the same for *Paramecium* in the two lots, although one was with heat filter, the other with no glass; both were run on the same day. There may have been a slight decrease in intensity of sunlight during the later part of the exposure, prolonging the lives of *Frontonia* and *Paramecium* individuals which had not yet cytolyzed, though no change was recorded. The range of survival times in Janus green 1/200,000 and Nile blue sulphate 1/500,000 is, in general, wider than with the other dyes. Comparison between indophenol and the dyes, as regards concentrations, is, of course, not possible. Bearing in mind that data from different cultures of mixed populations exposed on different days are included in the table, most of the data show rather high consistency for each species and each dye. Only a single indophenol lot is included in the table because indophenol, in the concentrations which are not rapidly toxic, is not very active photodynamically.

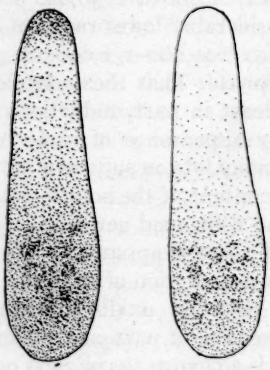
Control lots parallel to those of Table 1 and begun at the same time but kept in darkness, except for brief periodic examinations without exposure to strong light, all remained completely normal for at least 2 hours, when observations on some lots were necessarily concluded. Other lots in darkness, observed for longer periods, were normal after 3–4 hours, even in Janus green and Nile blue sulphate. In the concentrations of methylene blue of Table 1 most *Paramecium* individuals live for a day or more; *Fron-*

tonia and *Spirostomum* individuals, mostly for somewhat shorter periods.

Many other lots were run for photodynamic effects, some with different degrees of staining in darkness before exposure to sunlight, some with reduced dyes, some in diffuse daylight with clear or dark cloudy sky and with or without window glass intervening. Animals more or less stained in darkness before exposure to full sunlight are more sensitive than those of Table 1 and cytolyze at times ranging from a few seconds to a few minutes, according to dye and depth of stain. Photodynamic toxic effects and cytolysis occur, even in rather dim diffuse daylight, though, of course, less rapidly and with a much wider range of survival time than in full sunlight, but more rapidly than in controls in darkness, within a wide range of dye concentrations. With high dye concentrations cytolysis in diffuse light may result from the dye or indophenol before photodynamic effect is appreciable. With very low concentrations photodynamic effects in diffuse light may be slight or inappreciable.

To animals colored by indophenol or stained by the oxidized dyes, light is evidently an irritant or stimulant. Locomotion becomes more rapid; the tendency to dense aggregation increases, most conspicuously in *Paramecium* but also in the other forms; and in strong light there is an increase in frequency or continuation of backward locomotion. Accompanying this increase in activity there is more rapid posteropetal decoloration, or primary absence of color extends farther posteriorly and occurs in higher concentrations without oxygen decrease, even when small numbers of individuals are spread on slides in thin films of solution so that high oxygen concentration is assured. With deeper staining, anterior decoloration may be retarded, as in Figure 11. Even in full sunlight the longer-living individuals may show some posteropetal decoloration or failure to stain before toxic photodynamic effects appear. In diffuse daylight these modifications may become more conspicuous because of the longer period preceding toxic light-effects. In *Paramecium* these indirect differential effects of light appear in greater or less degree in a wide range of concentrations of methylene blue and neutral red from 1/20,000 or 1/30,000 down and in a considerably lower range of concentrations, 1/200,000–1/2,000,000, of Nile blue sulphate. That these decolorations are, at least in part, reductions is indicated by appearance of some recoloration in many lots on sufficient increase of oxygen content of the solutions by thorough and continued aeration or by oxygen, but there is apparently also an increase in destruction of the dyes. The effect of light on oxidized intracellular Janus green is of particular interest because it is a change to colorless or an approach to colorless with no trace of the red, partially reduced stage which appears with low oxygen, but may be partially reversed by oxygen in some cases. It may be entirely posteropetal or, in animals more deeply stained before exposure to light, posteropetal except for retardation anteriorly. Some *Paramecium* individuals may become almost completely colorless, but the decoloration usually does not progress so far posteriorly as with the other dyes before it is inhibited by toxic effects. Figures 29 and 30 indicate characteristic decoloration patterns. The decoloration has been observed in concentrations of 1/200,000–1/800,000. With sufficient increase of oxygen in the solution there may be more or less recoloration to blue-green, again without the red stage. In *Frontonia* there is less difference in light and darkness than in *Paramecium* as regards decolora-

tion and primary absence of color anteriorly; but these effects of light do appear, more clearly in diffuse light than in sunlight in most cases, less rapidly with Janus green than with other dyes and without a trace of red. *Spirostomum* is susceptible to light with little or no appreciable stain, but by intracellular oxidation of the leucobases of methylene blue and Nile blue sulphate it can be rapidly stained. After such staining more



29 30

FIGS. 29 AND 30.—*Paramecium:* partial decoloration of Janus green without red stage in light. Fig. 29, decoloration posteropetal as far as it takes place, except for retardation by deeper staining anteriorly. Fig. 30, with less staining decoloration, completely posteropetal.

rapid posteropetal decoloration has been observed in diffuse light with methylene blue 1/100,000 and lower and with Nile blue sulphate 1/200,000 and lower. After $2\frac{1}{2}$–3 hours in oxidized Janus green 1/100,000, most individuals are slightly deeper in color anteriorly. Half of such a lot exposed to diffuse daylight shows distinctly more rapid and more complete decoloration from green to colorless without any red than does the other half in darkness. Decoloration is clearly posteropetal from the anterior end, often with the short anteropetal gradient posteriorly. The posteropetal decoloration gradient is still visible after shortening, and in many individuals an almost spherical form results from toxic effects. In *Frontonia* and *Spirostomum*, as in *Paramecium*, the decoloration of Janus green without the red intermediate stage is to some extent reversible by high oxygen, and to that extent appears to be reduction. These color changes of Janus green in light are of particular interest because they appear to be, in part or entirely, reduction and reoxidation without appearance of the red partially reduced stage. A similar reduction appears with KCN in light (p. 291). There may also be some gradual destruction of the dye as well as reduction. Recoloration may not attain the original depth of color before exposure to light. Intracellular red, partially reduced Janus green may also be decolorized in diffuse light if the concentration is low, so that toxic photodynamic effect does not appear too soon. Since these color changes of Janus green in light appear to be in part or entirely reduction in the usual oxygen concentrations of preparations open to air or in thin film of solution, and since some reoxidation may occur on increase of oxygen, they raise the question whether the redox potential is increased by light to such an extent that the same reducing system is effective in light without oxygen decrease as in darkness with low oxygen or whether a different reducing system may perhaps be concerned in the light-effects. The intracellular dye concentrations are, of course, very low; and nothing is known concerning intracellular oxygen concentration. Whatever the factors concerned in these effects of light, it is a point of some interest that the gradient pattern appears in them as definitely as elsewhere.

One further point concerning photodynamic action on these ciliates is of interest, though only indirectly related to

gradient pattern. When intracellular indicators are reduced to colorless, the animals live much longer in light, even in sunlight, than animals stained with the same, or considerably lower, concentrations of oxidized dyes. If the reduced animals are completely colorless, they usually live until killed by lack of oxygen, except *Spirostomum*, which is more sensitive to sunlight than the other forms when not appreciably stained. In these experiments the animals are stained in darkness by oxidized dyes; then part of the lot is sealed with numerous animals in small volume of dye solution for reduction by oxygen uptake. Both the sealed and the open portions of the lot remain in darkness until reduction in the sealed lot has reached the desired point. When that stage is reached, the reduced portion and an equal volume of the oxidized portion on a depression slide, under cover like the reduced portion, are exposed to light. Sunlight has been found most satisfactory in these experiments because in diffuse light the animals stained with oxidized dye may undergo more or less decoloration, and the reduced animals may be affected by lack of oxygen before photodynamic effect is evident. Hydrosulphite has been used for reduction in some cases, but in sealed preparations after reduction by hydrosulphite *Paramecium* and *Frontonia* individuals are soon injured or killed by lack of oxygen. A lot consisting of *Paramecium* and *Spirostomum* stained with methylene blue 1/50,000 will serve as an example of the results attained with this procedure. On exposure of the oxidized and reduced portions of the lot to full sunlight the difference was striking. In the oxidized portion all *Spirostomum* individuals were completely disintegrated, and 40–50 per cent of the paramecia were cytolyzing within 3 minutes, 99 per cent or more within 8 minutes, and all within 13 minutes. In the reduced portion *Spirostomum* individuals disintegrated in 5–8 minutes, and 99 per cent or more of the paramecia were still normal and colorless after 13 minutes but with motor activity somewhat decreased by lack of oxygen. This portion was then opened and aerated with rapid oxidation and recovery of motor activity, but with less color than originally, presumably in consequence of destruction of dye. On exposure to sunlight again, an estimated 30 per cent cytolyzed within 3 minutes, 50–60 per cent within 12 minutes, most of the others were slow or motionless, and those still normal in form and activity were decolorizing posteropetally, although repeatedly aerated, and consequently were becoming less susceptible to light. *Frontonia* stains so much more rapidly with methylene blue than does *Paramecium* that somewhat lower concentrations of dye or shorter staining periods show most clearly the differences in photodynamic susceptibility with oxidized and reduced intracellular dye. Essentially similar results were obtained with Nile blue sulphate and neutral red. Even *Spirostomum*, though showing no appreciable color, except in food vacuoles and the contractile vacuole, disintegrated completely in 1–2 minutes in oxidized neutral red 1/60,000 or 1/50,000 in full sunlight and remained unaffected after 10 minutes in the same concentration reduced.

With intracellular Janus green reduced to the red stage, all three ciliates live slightly longer in sunlight than with oxidized dye if staining is very slight; but with deeper staining, they die earlier. For example, after 15–20 minutes in Janus green 1/100,000 or 1/200,000 in darkness, the solution was diluted ten times or more, so that little increase in intracellular concentration was possible, even after reduction; then half was re-

duced to red by hydrosulphite, the other half remaining oxidized. In sunlight the slightly red, partially reduced animals lived a little longer than the fully oxidized portion. In other lots with less rapid reduction to red by oxygen uptake of the animals in sealed preparations without dilution after 15 minutes in oxidized Janus green 1/1,000,000 or 1/2,000,000, the animals reduced to red, but only slightly colored, lived longer than the portion slightly stained with oxidized dye. With deeper staining by Janus green the red color of the partially reduced dye appears much more intense than the rather pale greenish of the oxidized stain. Apparently the intensity of the red color after deeper staining and reduction is a factor in the earlier death of the red- than of the green-stained animals in light.

Reduced intracellular indophenol has little or no photodynamic effect. A lot of *Paramecium* consisting of a large number of animals sealed immediately after addition of reagents in standard concentration showed no reaction after 15 minutes in darkness; a similar lot, also in darkness but open in the same concentrations, at the same time showed medium deep reaction after 15 minutes. In sunlight on the microscope stage 80–90 per cent of the oxidized lot were cytolyzed or cytolyzing, the reduced lot all normal after 20 minutes, still normal, but affected by lack of oxygen, after 30 minutes. On opening and aeration, oxidative recoloration occurred in a few seconds; and after 5 minutes more in sunlight 70–80 per cent were cytolyzed or motionless. This lot is much more sensitive to light after reoxidation than the lot in Table 1 with double the concentration of reagents and the oxidized lot in this experiment. Possible factors in this high sensitivity are: the longer exposure to indophenol reagents and the higher intracellular concentration of indophenol, aftereffect of lack of oxygen or perhaps of light during the reduced period, and the rapid intracellular oxidation of indophenol. Rapid oxidation is probably the chief factor; both indophenol and dyes show relatively high toxicity with rapid intracellular oxidation.

In general, effects of lack of oxygen on *Paramecium* and *Frontonia* in reduced lots make it difficult to determine whether they are more susceptible to light after intracellular reduction of indicators than animals without indophenol reaction or staining. They seem to be somewhat more susceptible. *Spirostomum*, although not visibly affected by lack of oxygen in the reduced lots, is apparently also somewhat more susceptible to light after reduction of intracellular indophenol or dye.

INDOPHENOL REACTION AND STAINING IN RELATION TO PH

In connection with a study of the effect of KCN on action of the indicators, it appeared desirable to examine effects of bases and acids on indophenol reaction and staining. Results of interest, obtained almost entirely with *Paramecium* material, are presented here. The pH range in the yeast cultures is approximately 7.6–8.0. Regions of a culture near the bottom, where the animals are more numerous and often more or less aggregated, are usually nearer the lower limit of the range than regions free from the animals and near the surface. The pH has been increased by bringing culture water containing the animals to various, not visibly toxic or only slowly toxic concentrations of the strong bases KOH and NaOH and the weak base NH_4OH and by comparing indophenol reaction and staining with that in controls in culture water.

With very slight increases of pH by

small amounts of KOH or NaOH the indophenol reaction is slightly accelerated, and the reaction gradient is usually somewhat steeper; also, the toxic effect of indophenol appears earlier. However, with increase of pH above approximately 8.5, retardation of reaction begins to appear, becoming greater with further increase of pH. At a pH which retards the reaction slightly animals may live a little longer than controls, because toxic effect of intracellular indophenol is delayed. Concentrations of NH_4OH which increase pH 0.3–0.4 may accelerate the reaction slightly in its early stages, but toxic effects of the ammonia appear soon and retard further reaction. In a few lots of *Frontonia* with increased pH only retardation of reaction, in *Spirostomum* only marked retardation and complete absence of reaction in a larger percentage of individuals than in controls, has been observed. From the data it appears that the upper limit of acceleration of reaction in *Paramecium* by increase of pH is not much above the pH of the cultures; in *Frontonia* and *Spirostomum* it appears to be lower than in *Paramecium*. These experiments were undertaken primarily in the hope that they might aid in interpreting effects of KCN on the indophenol reaction in *Paramecium;* KCN in solution gradually decomposes into KOH and HCN. Their relation to the effects of KCN will appear later. They present other problems which are outside the field of the present study and require extended experiment with different ranges of pH. With decrease of pH by means of dilute HCl or acetic acid to neutrality or slightly below, the indophenol reaction is much less rapid than in controls; and the posteropetal gradient is less steep than in controls, is absent, or the reaction gradient is anteropetal, as in Figures 4, 5, and 7. With the same decrease of pH in the solution, acetic acid is more effective in retarding the reaction than HCl, presumably because it penetrates more rapidly.

Staining of *Paramecium* by the oxidized dyes in relation to pH presents certain points of interest. With methylene blue, rate of staining increases with increase of pH until the pH becomes rapidly toxic and lethal. Also, the posteropetal ectoplasmic gradient becomes steeper than in controls and similar to the pattern of indophenol reaction (see Figs. 1–3); or it may become even steeper, with anterior ectoplasm almost opaque black with dye while the animals are still active. In general, a given concentration of methylene blue with increased pH stains like a much higher concentration in culture water, that is, more rapidly and with less or no decoloration, and the staining gradient usually becomes steeper. For example, with methylene blue 1/100,000 in culture water, color usually appears first in the posterior entoplasm; the posterior ectoplasm stains very gradually with anteropetal gradient, and the progress of stain anteriorly is very slow, anterior ectoplasm often remaining colorless indefinitely. With the same concentration of dye and increased pH, color is visible over the whole cell in a few seconds; and in a few seconds more it becomes progressively deeper in the anterior ectoplasm than elsewhere, with a distinct posteropetal gradient. A concentration of 1/20,000 in culture water still stains slowly and is not toxic until the stain becomes deep after an hour or more. With high pH, staining of the whole cell is almost instantaneous; the posteropetal staining gradient becomes evident almost at once; the anterior ectoplasm becomes opaque black with dye; and cytolysis begins within a few minutes. With high pH, intracellular accumulation of dye is evidently so rapid that destruction or reduction of dye oc-

curs only in much lower concentrations than in controls. With Janus green the effect of increase of pH is, in general, similar but appears to be less in degree. Staining is somewhat more rapid in all parts and relatively more rapid in anterior ectoplasm, and toxic effect and cytolysis occur earlier than in controls. Similarly with Nile blue sulphate, rate of staining and steepness of staining gradient are increased with increase of pH, but visible difference from controls is less than with methylene blue. With neutral red the effect of increase of pH is difficult to distinguish with certainty. Rate of general staining is apparently slightly increased; and in the earlier stages of staining a very slight staining gradient seems to be more clearly, or more frequently, visible than in controls. As regards rate of general staining, it may be suggested that increase of pH increases permeability to the dyes. So far as absolute concentrations are concerned, Nile blue sulphate and neutral red penetrate more rapidly than methylene blue and Janus green. Exact comparisons are impossible, but a given concentration of methylene blue in culture water appears to stain no more rapidly than concentrations of Nile blue sulphate, and neutral red a fifth of that. Janus green in low concentrations, made necessary by its high toxicity, also appears to stain less rapidly than do Nile blue sulphate and neutral red, but the rather faint color of oxidized Janus green in the cells makes comparisons still more uncertain. Apparently, increase of pH increases rate of general staining by the more rapidly penetrating dyes, Nile blue sulphate and neutral red, less than by the less rapidly penetrating methylene blue.

The increase in steepness of the posteropetal staining gradient—that is, the relatively more rapid staining of the anterior ectoplasm with increase of pH—raises the question whether differential permeability or other factors are involved. No conclusive evidence of differential permeability has been found in the course of this study. The earliest stages of staining in culture water and with increase of pH seem to be uniform over the whole cell, the staining gradient appearing only gradually and becoming progressively steeper. The gradient is more directly dependent on dye concentration than on pH and increases in steepness with increasing concentration of dye, at least within a certain range; with very low concentrations it is absent or reversed, anterior regions remaining unstained. It is similar to the indophenol reaction gradient and the oxidation gradient of reduced indophenol and dyes and shows the same modifications as these. In short, it appears to be an expression of the physiological gradient pattern, somewhat modified by increase of pH. If it is associated with a differential increase of permeability with increase of pH, that differential is itself evidently correlated with the oxidation-reduction pattern.

Decrease of pH slightly below neutrality by hydrochloric or acetic acid decreases rate of staining and decreases, obliterates, or reverses the staining gradient. Even with neutral red the ectoplasm stains less rapidly over its whole length than in controls and usually shows a slight anteropetal gradient; the entoplasm shows no appreciable difference from controls. Questions concerning the relation of these effects to permeability arise here, as with increase of pH. In general, a given dye concentration with low pH stains essentially like a lower concentration in culture water, with reversal of the staining gradient in consequence of posteropetal decoloration or failure of anterior regions to stain, as indicated in Figures 4, 5, and 7.

Indophenol suggests differential increase and decrease of oxidase activity with increase and decrease of pH within certain limits. The staining pattern with the dyes shows essentially the same modifications with change of pH as the indophenol pattern, but less in degree with neutral red than with the other dyes. These data again suggest the possibility that staining patterns may be more or less closely related to patterns of oxidase activity in some way, irrespective of the presence or absence of differential permeability. If this is the case, the relation seems to differ in degree for the different dyes; and the question whether these dyes stain living protoplasm in the same way arises. The problem of the mechanism or mechanisms of "vital" staining is involved.

The pH of the cell body of *Paramecium* is also of interest, particularly in relation to the indophenol reaction and the effects on it of increase of pH and of KCN, and in relation to staining with neutral red, which is an acid-alkali, as well as an oxidation-reduction indicator. Neutral red has been used as an acid-alkali indicator with *Paramecium* by other investigators, but the validity of conclusions drawn from the staining color of this dye in the *Paramecium* body has been questioned. Here a few observations which appear to possess some significance are noted. In the earlier stages of staining of *P. multimicronucleatum* by oxidized neutral red the color indicates a pH near neutrality, perhaps very slightly on the acid side, even when the external medium is alkaline. Most of the recently formed food vacuoles and some other entoplasmic inclusions stain as if somewhat more acid than the ectoplasm (cf. Shapiro, 1927). It has been observed that animals with numerous food vacuoles from cultures with abundant food seem often to show slightly more acid color in early staining than those with few or no food vacuoles; but the difference is so slight, if real, that it is not regarded as fully demonstrated. In neutral red 1/50,000 or lower concentrations, more or less secondary posteropetal decoloration of ectoplasm occurs. This is due to reduction or destruction of the dye; but with the low ectoplasmic concentration of the dye and the less intense color of the yellow alkaline neutral red, the possibility that some increase in ectoplasmic pH takes place posteropetally cannot be entirely excluded. As the intracellular dye becomes toxic, the animals become somewhat swollen, evidently by uptake of water—often to such an extent that an entoplasmic vesicle is forced out of the anal region and may attain considerable size while the animals are still active. A distinct posteropetal ectoplasmic change in color toward yellow appears in these later stages of neutral red staining, and on approach or beginning of cytolysis a further color change to full yellow progresses posteropetally over the whole body; entoplasmic inclusions, particularly those in the interior of the postoral entoplasm, show this change last.

In the same concentrations of neutral red, slightly acidified by HCl, the color in earlier stages of staining is the same as in alkaline solution. With the earliest indication of toxic effect in retardation of locomotion, the color becomes posteropetally more acid and remains without further change as cytolysis occurs. Apparently, in both alkaline and acid solutions permeability to the external medium increases with toxic effect, the increase progressing posteropetally; or the ability to maintain a pH different from that of the solution decreases.

If *Paramecium* is normally neutral or slightly acid, as neutral red indicates, it would appear to be unfavorable material for the indophenol reaction, which

supposedly requires an alkaline medium. However, the reaction occurs rapidly and color becomes deep, even with the low concentrations of reagents used with the living material. The aniline solution is alkaline in reaction. One small drop of the stock aniline solution, added to 1 ml. of culture water (that is, the standard concentration for the reaction) increases pH 0.2–0.4, as indicated by phenol red. In neutral red 1/50,000 and the standard concentration of aniline (1/8,000 of the commercial sample), both added to a *Paramecium* lot at the same time, the earliest stages of staining are not distinguishable from controls in the same dye concentration without aniline. However, in from one to several minutes the lot with aniline shows a distinct change in color toward yellow, first in the ectoplasm and then progressing into the entoplasm; entoplasmic inclusions retain acid color after the entoplasmic ground substance has become yellowish but may finally become more or less alkaline in color. The animals may become entirely yellow while still normal or with only slight evidence of toxic effect. Controls show no such color change until they cytolyze, although, as noted above, they do become somewhat more alkaline in color posteropetally in later stages of staining. With twice the standard concentration of aniline the color change is more rapid and extreme. Apparently, *Paramecium* is able to neutralize the very low intracellular concentration of aniline for a short time after exposure; but the concentration soon becomes too high for the neutralizing capacity of the animals, and the change in color begins. A lot of *Paramecium* in standard aniline concentration for 15 minutes before addition of neutral red stains almost completely yellow from the beginning, in extreme contrast with the red-staining control.

Aniline-neutral red lots made slightly acid by a trace of acetic acid after alkaline staining become red almost at once; but the color is somewhat less deep than in controls and increases posteropetally, again suggesting a differential destruction of neutral red or a differential retardation of staining by the presence of aniline, to which *Paramecium* is differentially susceptible.

These experiments show, beyond question, that aniline in the concentrations used for the indophenol reaction increases the protoplasmic pH of *Paramecium* before toxic effects appear. The reaction is greatly retarded when the protoplasmic pH is decreased by acetic acid (p. 283). Evidently the alkaline aniline, by increasing somewhat the protoplasmic pH, renders intracellular conditions more favorable than they would be with the natural intracellular pH of the animals. As noted above, animals gorged with food often appear slightly more acid in color in earlier stages of neutral red staining. Such animals very commonly show an indophenol reaction resembling that in neutral or slightly acid culture water. The reaction is less rapid, the gradient is less distinct, and death occurs with less color than in more favorable material. Apparently, in these cases the alkalinity of the aniline does not bring the protoplasmic pH to the point most favorable for the reaction. Retardation of the indophenol reaction by increase of pH above a certain point in the culture water indicates that under these conditions protoplasmic pH becomes too high for the most rapid reaction.

THE EFFECTS OF POTASSIUM CYANIDE

The effects of KCN on the indicator pattern have been studied in several hundred experimental lots under various conditions, almost entirely with *Paramecium* as material and with both indo-

phenol and dyes. *Paramecium multimicronucleatum*, like *P. caudatum*, is not highly cyanide-sensitive; animals from various cultures have remained normal and active for an hour in alkaline KCN m/100 in closed containers with small air space. Different cultures and individuals of the same culture differ considerably in sensitivity. After 48 hours in KCN m/1,000 there is little or no visible effect.

The question whether KCN enters living cells as KCN or as HCN has been raised by earlier investigators. Neutral red, being a pH, as well as an oxidation-reduction, indicator, throws an interesting light on this question. Experimental lots of *Paramecium* brought into various concentrations of KCN from m/1,000 to m/100 with various concentrations of neutral red, e.g., 1/50,000, show in the earliest moments of staining no appreciable difference from those in neutral red 1/50,000 without KCN. However, within a few minutes a change in color from red toward the alkaline yellow of neutral red begins to appear anteriorly in KCN-neutral red lots and progresses posteriorly over the whole cell body, except that food vacuoles and other entoplasmic inclusions stain as if acid, as they do in absence of KCN. In m/1,000 the change in color is not very great; in m/200 with high neutral red concentration it becomes very distinct, the whole cell body, except the interior of the postoral entoplasm, becoming yellowish posteropetally, while controls in neutral red alone remain red. As oxidase activity is progressively inhibited by the cyanide, the animals begin to reduce the dye, even without decrease of oxygen (p. 290); and the yellowish neutral red gradually becomes colorless posteropetally. With addition of neutral red after 15–30 minutes in KCN m/200 or m/100, the lower concentrations, e.g., 1/40,000, are reduced to colorless as rapidly, or almost as rapidly, as the dye enters; and only entoplasmic inclusions, chiefly postoral, show more than very slight color. In these cases neutral red acts first as pH indicator and later as oxidation-reduction indicator. As the cyanide-dye solution becomes toxic, reduction of neutral red is gradually inhibited and the animals become yellowish, in the course of cytolysis becoming completely yellow.

Potassium hydroxide, in various, not rapidly toxic concentrations, produces no visible change in intracellular color until cytolysis is about to begin or is beginning. Apparently it does not penetrate or penetrates so slowly that it is neutralized as rapidly as it enters until the cell surface is injured. Even though the external KOH–neutral red solution is completely yellow, the animals stain the same red color as with neutral red in culture water. Since KOH alone produces no visible color change, the color change with KCN suggests that it enters the *Paramecium*, at least in part, as molecular KCN and decomposes into KOH and HCN within the cell and that the KOH thus formed within the cell is sooner or later not completely neutralized, increases intracellular pH slightly, and so alters the color of neutral red within the cell. The posteropetal progress of the intracellular color change may conceivably be due to a permeability gradient to cyanide, to a slight posteropetal decrease in the natural intracellular pH, to a posteropetal decrease in rate of decomposition of cyanide, or to some other differential. Evidence of a very slight differential permeability to extremely low concentrations of weak bases and probably of weak acids was obtained by Child and Deviney (1926) with *P. caudatum* stained by neutral red. With higher, but still not rapidly toxic, concentrations, penetration appeared to be uniform over

the whole length of the cell and virtually instantaneous, as indicated by the color change of neutral red. That there is any appreciable difference in permeability at different levels of the *Paramecium* body to KCN m/200 or m/100 seems improbable, but the posteropetal gradient of color change of neutral red appears with these concentrations; it is apparently another expression of the physiological gradient pattern. These results with neutral red and KCN provide a starting-point for consideration of some other effects of KCN.

When animals remain in KCN m/1,000 for periods ranging from several hours to 2 or 3 days in closed containers with small air space before addition of indophenol reagents, the indophenol reaction is almost invariably more rapid than in controls in culture water and usually more rapid after longer, than after shorter, periods in KCN. Acceleration of reaction is greatest anteriorly; consequently, the posteropetal reaction gradient becomes more distinct—that is, steeper than in controls. After 1–2 hours in KCN m/500 the reaction is essentially similar in most individuals. With these concentrations and exposure periods, few or no individuals show any marked toxic effects, though locomotion may become slightly less rapid than in controls. The acceleration of the indophenol reaction indicates that the slight increase in intracellular pH overbalances any inhibiting action of cyanide on oxidase activity.

With exposure to KCN m/100 and indophenol reagents at the same time, reaction is usually somewhat accelerated in the anterior ectoplasm during earlier stages, as compared with controls with indophenol reagents in culture water; but it becomes progressively less rapid posteropetally and in some individuals may be slight or absent in posterior regions. Figure 31, primarily for cyanide-dye, indicates an extreme indophenol effect. It is suggested, first, that in these cases the reaction in the anterior region, where it is normally most rapid, is accelerated in its early stages by the slight increase in intracellular pH resulting from decomposition of KCN within the cell. Second, several factors may be concerned in the posteropetal apparent retardation or inhibition of reaction. Since reaction is normally less rapid in these regions than anteriorly, decrease of oxidase activity by KCN or HCN may become increasingly effective in retarding or decreasing the reaction; also, intracellular pH may become high enough to retard reaction before it becomes deep in the less rapidly reacting regions; and, finally, since reduction of intracellular indophenol may occur with little or no oxygen decrease in presence of cyanide (p. 289), there may be some reduction where oxidase activity is decreased before the reaction has progressed far. The retardation of intracellular oxidation of reduced indicators in the presence of cyanide (p. 292) indicates that decrease of oxidase activity by cyanide is the chief factor in retardation or inhibition of indophenol reaction, and that, when the animals are exposed to KCN and indophenol reagents at the same time, the slight increase of intracellular pH becomes effective before the inhibiting action on oxidase. Consequently, the pH effect appears anteriorly; the cyanide effect on oxidase, at more posterior levels. There is also some reduction of intracellular indophenol without decrease of oxygen; slight increase of color may occur on thorough aeration unless the reducing system is also injured by high concentration of KCN or long exposure.

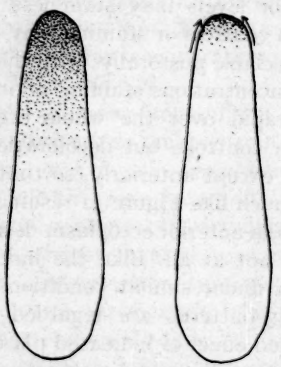
With exposure to KCN m/100 for periods ranging from a few minutes up to half an hour before addition of indo-

phenol reagents, reaction is less rapid or very slight throughout the cell; but, even in these lots, it may be less retarded in the anterior ectoplasm of at least some individuals than at more posterior levels. Here the cyanide effect on oxidase overbalances and may completely obliterate pH effect. With these procedures there is considerable quantitative variation, involving both rate and degree of effect, in individuals of the same lot and in lots from different cultures. However, the effects described have been observed with KCN in greater or less degree in many lots from many cultures and are believed to be characteristic for the different experimental conditions with newly made KCN solutions and animals apparently in good condition from yeast cultures.

With exposure to KCN m/500 or higher following indophenol reaction, reduction with oxygen decrease by hydrosulphite or by oxygen uptake of the animals in sealed preparations is more rapid than in controls. It also occurs to a much greater degree without experimental oxygen decrease, particularly if individuals are numerous or if there is any aggregation. It is posteropetal, as in Figure 10, unless the anterior reaction has progressed so far that reduction is retarded there by early toxic effect, as in Figure 11. Reoxidation is retarded or completely inhibited by cyanide until the dehydrogenase system is also damaged. Susceptibility to cyanide decreases posteropetally; consequently, the damage progresses posteropetally, and, as it progresses, some degree of reoxidation occurs, also posteropetally, and cytolysis soon follows.

With the dyes, patterns of staining, reduction, oxidation, and reoxidation may also be altered by KCN. With methylene blue and Nile blue sulphate the alterations are very similar to those with indophenol, though the factors concerned are apparently not completely identical. With exposure at the same time to KCN m/500–m/100 and methylene blue 1/30,000–1/50,000 or Nile blue sulphate 1/200,000 or lower, staining of the anterior ectoplasm is more rapid, and the staining gradient is steeper, than in controls. The anterior region may become more rapidly and more deeply colored, as in Figure 31. More posterior levels may stain less rapidly than in controls or staining may become inappreciable postorally; with the higher dye concentrations staining is primarily more rapid over the whole ectoplasm than in controls, but decoloration soon occurs, except anteriorly, so that conditions much like Figure 31 result secondarily. The anterior ectoplasm decolorizes last or not at all. Like the indophenol pattern under similar conditions, these staining patterns are regarded as the combined effect of increased pH and decrease of oxidase activity by cyanide. In consequence of increased pH in the solution and within the cells the anterior region stains rapidly and deeply, and the whole ectoplasm may stain before the more gradual decrease of oxidase activity by cyanide is effective in permitting reduction of the dye, except that in the anterior region staining has become so deep that reduction is usually retarded or completely inhibited there, while still possible further posteriorly. If the anterior region is not too deeply stained, it may gradually reduce anteropetally, the extreme tip reducing last or remaining deeply stained (Fig. 32). That absence of stain posteriorly, as well as secondary decoloration of these regions, is due chiefly or wholly to dye reduction, rather than to retardation of staining or destruction of dye, becomes evident on increase of oxygen in the solution. With thorough and continued aeration the animals become more deeply colored

throughout than controls. Increase of oxygen antagonizes the decrease of oxidase activity by cyanide. Since the animals tend to remain close to the bottom of the solution, they soon decrease the oxygen content there sufficiently, if they are numerous and are left undisturbed, to reduce the dye again, unless intracellular accumulation of dye was sufficient to become toxic on oxidation. Aeration often brings about cytolysis in the anterior region while the rest of the body is still able to reduce. As toxic effect of the dye-cyanide solution develops, the reducing system is inactivated; intracellular dye, like indophenol, gradually reoxidizes in spite of the presence of cyanide, and death follows.



FIGS. 31 AND 32.—*Paramecium:* Fig. 31, rapid and deep staining of anterior region with exposure at same time to KCN m/500–m/100 and methylene blue or Nile blue sulphate; also, extreme cyanide effect on indophenol reaction; the rest of the body may remain almost or quite without color in consequence of dye reduction and of retardation of indophenol reaction and reduction of indophenol. Fig. 32, anteropetal reduction of anterior region with KCN and less deep reaction or staining; extreme anterior tip reduces last or not at all.

With exposure to KCN for various periods before addition of methylene blue or Nile blue sulphate, staining is less rapid; the staining gradient is less steep or is obliterated, or reduction may be completely posteropetal as in uninjured animals with low oxygen. With longer exposure to KCN or higher concentrations, color may be almost completely absent, until the reducing system is inactivated by toxic effect. That these alterations of staining pattern are due largely or entirely to reduction is shown by the immediate increase of color on increase of oxygen in the solution; the KCN lots become more deeply stained than controls. With exposure to KCN before dye, decrease in oxidase activity, permitting reduction, overbalances any visible effect of increased pH until the deeper staining appears with oxygen increase.

With exposure to neutral red and KCN m/200–m/100 at the same time, there is no more effect of increased pH on rate of staining than with KOH (p. 284). The anterior region does not become appreciably more deeply colored, but a posteropetal color change toward the alkaline yellow of the dye begins within the first few minutes and is apparently followed by posteropetal reduction to colorless, though, as the color becomes more alkaline, it appears fainter, and it is somewhat difficult to distinguish color change from reduction in the earlier stages. Anterior regions usually become colorless; posterior regions reduce less completely. No retardation of reduction in the anterior region has been observed with neutral red, but with change from red toward the alkaline yellow it is difficult to determine whether the anterior region is more deeply stained. Moreover, the staining gradient with neutral red, either in culture water (p. 267) or with increase of pH (p. 284), is very slight. Under these conditions the effect of increase in internal pH appears first; the effect of cyanide, more gradually. The reductions in the cyanide–neutral red lots occur in open preparations entirely

without experimental oxygen decrease. With addition of neutral red after 15–20 minutes in KCN m/200 or m/100, visible staining is very slight and may be limited to the postoral entoplasm, and reduction may become complete if toxic effect does not prevent.

In Janus green 1/400,000–1/200,000 and KCN m/500–m/100, staining progresses slightly more rapidly; the staining gradient usually appears slightly steeper; and cytolysis occurs earlier than in controls in preparations kept in darkness except for examinations. The macronucleus becomes reddish in color, indicating partial reduction; and in some individuals a very slight shift in color toward reddish seems to be visible, except in the more deeply stained anterior region. These are the only evidences of reduction of Janus green by cyanide without experimental oxygen decrease; partial reduction in the macronucleus is distinct, but the other evidences of reduction are somewhat doubtful. On exposure to strong diffuse daylight, reduction of Janus green directly to colorless, as described in an earlier section (p. 279), occurs to some extent in KCN–Janus green, though usually less than in controls because of earlier toxic effect. This, of course, is an effect of light not of cyanide; and the dye-KCN lots are, or soon become, less capable of showing it. Here, again, the question arises whether a reducing system different from that determining the reduction through red to colorless may be involved. With thorough aeration both dye-KCN lot and control become deeper blue-green with no trace of the red stage, the former deeper than the latter; and the partially reduced macronuclei are oxidized. With various periods in KCN before addition of the dye and with staining before addition of KCN, results are much the same until altered or inhibited by toxic effects. Staining is somewhat more rapid in KCN than in controls; reduction to reddish occurs in macronuclei, with perhaps a trace of reduction elsewhere. Janus green differs from the other dyes used in that there is no conclusive evidence of its reduction by cyanide without experimental decrease of oxygen, except in the partial reduction of the macronucleus. Apparently, decrease of oxidase activity by cyanide does not activate the reducing system sufficiently for general reduction of Janus green in preparations open to air and without experimental decrease of oxygen. The deeper staining by Janus green in presence of cyanide than in controls is regarded as due to the increase in internal pH.

Solutions of the oxidized dyes and KCN are more rapidly toxic and lethal than the same concentrations of dye or KCN alone; but if considerable reduction occurs, a dye-KCN lot may live longer than controls in dye or KCN unless concentration of KCN is so high that it kills before the dye becomes toxic. With reoxidation of dye reduced in consequence of decrease of oxidase activity by cyanide, toxic effect and cytolysis may occur almost at once, as with oxidation of the higher concentrations of reduced dyes alone. Appearance of this rapid increase in toxicity of dyes on reoxidation with lower concentrations of dyes with KCN than in controls with dye alone is believed to be due to more rapid intracellular accumulation of dye in the presence of KCN in consequence of increase of pH and perhaps also of intracellular reduction.

Animals exposed to KCN after staining by any of the four dyes reduce much more rapidly than controls and with less or no experimental oxygen decrease. In darkness with decrease of oxygen by hydrosulphite or by oxygen uptake of the animals Janus green reduces through

the red stage to colorless. Reduction is posteropetal, unless retarded or inhibited by deep stain (see Figs. 10 and 11). With KCN m/1,000 the effect is slight or inappreciable; in concentrations of from m/500 to m/100 it is much more distinct. Reduction may occur in animals swimming in the deeper parts of the 1-ml. volumes on large depression slides open to air, particularly if the number of individuals is considerable or if they are allowed to aggregate. Under these conditions reduction of Janus green progresses only to the red stage, may occur only at the more anterior gradient levels, is less frequent than with the other dyes, or may be absent, according to number of individuals and degrees of aggregation.

It is evident that action of cyanide is very similar to that of low oxygen, so far as reduction of intracellular indophenol and the dyes, except Janus green, is concerned. This is interpreted as resulting from decrease or inhibition of cytochrome oxidase activity, with the result that the animals are less able than the controls, or unable, to use the oxygen present. Under these conditions the dehydrogenase system reduces the indicators in the presence of higher oxygen concentration than in controls. The inhibiting action of cyanide on oxidase activity is distinctly posteropetal; reduction progresses posteropetally, except when retarded or inhibited anteriorly by accumulation of indophenol or dye in the anterior region. Even then, it is usually still posteropetal at more posterior levels, as in Figure 11. The data on reduction in the presence of cyanide are in complete agreement with the indophenol reaction and with rapid oxidation of reduced dyes (p. 264) in indicating a posteropetal decrease in oxidase activity—at least in the ectoplasm.

The most striking, directly visible evidence of decrease in oxidase activity in *Paramecium* by cyanide appears in intracellular oxidation of leucobases of the dyes. In these experiments the animals are exposed to KCN for certain periods, and their rate of oxidation of a dye leucobase is then compared with that of controls in culture water. For example, an experimental lot is brought into KCN m/200 or m/100 for 15–20 minutes. At the end of this period 1 ml. of dye—methylene blue or neutral red 1/20,000–1/50,000, Nile blue sulphate 1/100,000–1/200,000, or Janus green 1/50,000–1/200,000—is completely reduced by sodium hydrosulphite, care being taken to avoid excess of the reducing agent. Before reoxidation the reduced dye solution is separated into two equal parts; to one of these a drop of the lot in KCN, to the other a drop of animals in culture water as control, is added. In the control, intracellular oxidation becomes visible in a few seconds to a minute or two, depending on dye concentration and amount of hydrosulphite added, as described in an earlier section (p. 264), and the oxidation gradient is posteropetal, that is, oxidase activity decreases posteropetally. In the KCN lot there is little oxidation after 5–15 minutes, again depending on dye concentration and degree of cyanide effect. Even though the solution oxidizes, there may be little intracellular oxidation. In so far as oxidation does become visible during this time, it usually appears first in the postoral entoplasm, then in the posterior ectoplasm, and progresses anteropetally; that is, oxidase inhibition by cyanide decreases posteropetally. This differential parallels differential susceptibility to cyanide, as indicated by differential cytolysis. If the cyanide has not produced irreversible injury, it is so highly diluted in the leucobase solution that some degree of recovery with further oxidation may gradually take place. Incidentally,

it may be noted that, if the leucobase solutions for the two lots are made up separately, there is no certainty that they contain the same concentration of hydrosulphite; otherwise, exposure to KCN might be continued through the experiment. As in other KCN experiments, the inhibition of oxidase by cyanide, if not extreme, is antagonized, in some degree, by an increase of the oxygen content of the solution. After a given exposure period and concentration of KCN, the partial oxidation to red of completely reduced Janus green is somewhat more rapid than the complete oxidation of the other dyes, except after extreme action of cyanide; this is perhaps due to the low rH of this dye. With neutralized or slightly acid KCN there is a similar decrease of oxidase activity, though, with a given concentration of KCN before neutralization and a given exposure period oxidase inhibition seems to be less than with alkaline KCN. When KCN solutions are brought to the same pH as a particular sample of culture water (7.6–8.00) and when both KCN and culture water are brought to pH 7 or slightly below, *Paramecium* stains less rapidly in the KCN than in the control in culture water. With neutral red the difference is less than with the other dyes. If the animals remain for 5 minutes in KCN (e.g., m/200) before addition of dye, the difference from the control is greater, at least in earlier stages of staining. Here, again, the question of a possible relation between vital staining and oxidase activity arises. In the KCN solutions with decreased pH the decrease of oxidase activity is not overbalanced by high alkalinity. The decrease in rate of staining under these conditions, as compared with controls in culture water at the same pH, is differential, being greatest in the anterior ectoplasm, where the greatest oxidase activity is indicated, and decreasing posteropetally.

OTHER CILIATES

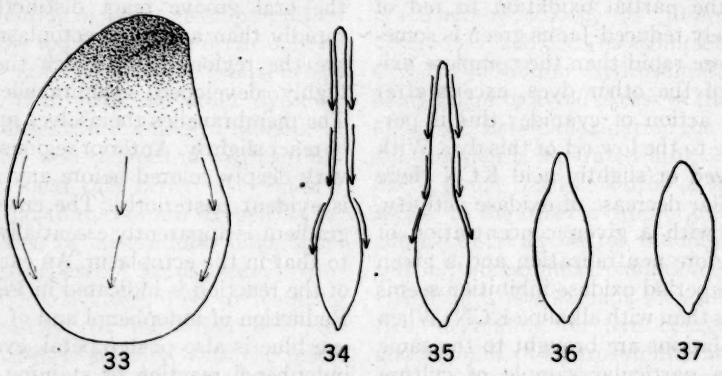
A few data on oxidation-reduction patterns of other ciliates are noted. In the large *Woodruffia metabolica* (Johnson and Larson, 1938) the indophenol reaction gradient is very distinctly posteropetal, the reaction is less rapid, and the gradient is much steeper, than in *Paramecium* and *Frontonia*. The margins of the oral groove react distinctly more rapidly than adjoining ectoplasm. They are the regions from which the rather highly developed membranelles arise. The membranelles themselves apparently react slightly. Anterior regions become very deeply colored before any reaction is evident posteriorly. The entoplasmic gradient is apparently essentially similar to that in the ectoplasm. An early stage of the reaction is indicated in Figure 33. Reduction of indophenol and of methylene blue is also posteropetal, even after indophenol reaction or staining is very deep anteriorly. Figure 33 serves to indicate the course of reduction if the shading and arrows are regarded as representing the reduction pattern rather than the indophenol reaction pattern. The margins of the oral groove reduce more rapidly than adjoining ectoplasm. This differential is often still visible in spherical, recently encysted individuals. As in *Paramecium*, rapid reoxidation is posteropetal until the anterior region is injured; slow reoxidation may be anteropetal.

A few individuals of *Lachrymaria* or a related form, appearing in cultures, show posteropetal indophenol reaction and reduction of indophenol and methylene blue more rapidly in the slender proboscis-like organ at the anterior end than in the rest of the body (Figs. 34 and 35). However, this organ, like the somewhat

similar organ of *Dileptus* (Child, 1934), is much more susceptible to indophenol reagents and dyes than is the rest of the body. In the higher concentrations a wave of complete disintegration passes posteropetally over its whole length in a few seconds but does not involve the rest of the body. In a lower range of concentrations the elongated organ gradually shortens and may disappear completely, being resorbed into the body without any disintegration or other evidence of exposure to experimental conditions, or sometimes even more rapid.

The indophenol reaction and reduction gradients are distinctly posteropetal in a species of *Colpoda*, apparently *C. cucullus*, as described and figured by Bütschli. For the reaction concentrations of reagents three or four times standard were found most satisfactory.

In the hypotrich *Euplotes*, indophenol reaction and indophenol and methylene blue reduction progress posteropetally.



FIGS. 33–37.—Oxidation-reduction patterns in other ciliates. Fig. 33, *Woodruffia metabolica:* posteropetal indophenol reaction gradient with more rapid reaction along margins of oral groove than in adjoining ectoplasm; assuming shading to represent reduction, the figure also indicates the posteropetal indophenol and dye reduction gradient and more rapid reduction along margins of the oral groove. Figs. 34–37, *Lachrymaria* (?): Figs. 34 and 35, posteropetal indophenol reaction gradient. Figs. 35–37, stages of dedifferentiation and resorption of anterior ectoplasmic organ.

cytolysis and while the rest of the body is not appreciably damaged (Figs. 35–37). In its normal form and extension it appears to be completely ectoplasmic; shortening and progressive resorption are accompanied by loss of motility and apparently constitute a real dedifferentiation. So far as could be determined from the few individuals available, it is in large part, or entirely, resorbed into the entoplasm rather than becoming a part of the ectoplasm. The slender anterior organ of *Dileptus* may undergo similar resorption and dedifferentiation (Child, 1934). In both cases the resorption may be complete in an hour after

Reaction and reduction appear to be most rapid along the oral groove or its margins bearing the membranelles. In this form with ventrodorsal differentiation the ventral ectoplasm from which the large cirri arise reacts and reduces more rapidly and with a more distinct posteropetal gradient than the dorsal ectoplasm. Cirri and membranelles apparently show a very slight indophenol reaction.

CONCLUSION

Extended discussion seems unnecessary. The presence of a posteropetal oxidation–reduction gradient pattern in

the living ciliate protozoans included in this study is demonstrated. The indicators make this pattern and its modifications under different experimental conditions directly visible. In the reconstitution associated with fission the single gradient becomes two gradients, in consequence of the appearance of a new region of high oxidase activity just posterior to the fission plane and its development into the anterior region of the posterior member of the fission pair. Because of the presence of the gradient pattern the effects of external factors on the animals are, in general, differential. Susceptibility to injury by the more extreme effects of external factors and the ability to antagonize or equilibrate to less extreme effects are greater at the higher than at the lower levels of the oxidation-reduction gradient.

The directly visible evidence of differential decrease or inhibition of oxidase activity by cyanide in *Paramecium*, the only form used in this line of experiment, is of special interest in relation to earlier attempts to determine whether oxygen uptake of *Paramecium* is decreased by cyanide and requires some further consideration. As regards effect of cyanide on oxygen uptake in *Paramecium*, there is no agreement. Lund (1918) found no decrease with cyanide until cytolysis; according to Kalmus (1928), there is a marked decrease; Gerard and Hyman (1931), using a phosphate buffer which alone decreases oxygen uptake about 50 per cent, found no further decrease on addition of neutralized NaCN. Alkaline KCN added to the buffer solution often, but not always, produced some decrease. *Paramecium* was held to be cyanide-insensitive by Shoup and Boykin (1931). The more recent data of Boell (1942) show a marked decrease, and he concludes that the respiration of *Paramecium* does not differ in its cyanide sensitivity from that of many other organisms. Whatever the final conclusions concerning sensitivity of oxygen uptake to cyanide in *Paramecium*, there can be no question concerning its effect in decreasing the rate of oxidation of leucobases of dyes. Also, this decrease is differential, agreeing with the gradient pattern made visible in other ways. The decrease appears not only in alkaline, but also, though apparently in lesser degree, with neutralized or slightly acid KCN of the same concentration before neutralization. Moreover, as regards reduction of oxidized intracellular indophenol and dyes, the cyanide effect is essentially similar to that of low oxygen (p. 292). These data are not quantitative and give no information as to amount of decrease of oxidase activity. It may be that a relatively slight decrease of oxidase activity, which would have little effect on oxygen uptake, is sufficient to retard intracellular oxidation of leucobases and to permit intracellular reduction of oxidized indicators without experimental oxygen decrease. Also the cyanide-sensitive system may represent only a part of the total oxygen uptake, as in various other organisms. And, finally, nutritive condition of the material may perhaps have played some part in the lack of agreement concerning cyanide and oxygen uptake. There is considerable individual variation in evidence of oxidase activity by the indicators. In cultures with decreasing food supply, or when kept with little or no food for a few days, *Paramecium* certainly seems to show less oxidase activity, as indicated by the indophenol reaction, than well-fed animals. Some experiments have seemed to indicate less decrease of oxidase activity by a given KCN concentration in starved than in well-fed animals. However, extensive experiment along these lines has not been attempted, since this question

is outside the field of the present study, and since, lacking a quantitative basis, the few data are of uncertain value and at best only suggestive. However, it is perhaps worth mentioning in this connection that in the rhizopod *Pelomyxa carolinensis* oxygen uptake has been found to vary with amount of food present in the cytoplasm (Pace and Belda, 1944). Whether there is any similar relation between food and oxygen uptake in *Paramecium* seems not to have been determined. Quite aside from these questions concerning effect of cyanide on respiration, intracellular oxidation and reduction of indicators give directly visible evidence of differential decrease of oxidase activity by cyanide in *Paramecium multimicronucleatum*.

Mention has been made, at various points in the paper, of quantitative differences in individuals of the same culture and in different cultures, as shown by the indicators. That field has been left essentially untouched, but it certainly presents problems of interest. The gradient patterns can always be made visible; but rates and degrees of effects with particular concentrations of indophenol reagents and dyes differ. Effects observed with certain concentrations, as noted, should be regarded rather as examples than as exactly reproducible with the same concentrations under all cultural conditions. The incidental observations along these lines suggest that, even in different yeast cultures, as well as in different individuals of the same culture, considerable differences in oxidation-reduction activity may occur, with age of culture, kind and abundance of food, and perhaps other cultural or genetic conditions as possible factors. With other culture media relations of indicator concentrations and particular effects may again be different from those in yeast cultures.

SUMMARY

1. Intracellular formation, reduction, and reoxidation of indophenol; intracellular reduction and reoxidation of the oxidized dyes methylene blue, Nile blue sulphate, Janus green, and neutral red; and primary intracellular oxidation of their leucobases demonstrate an oxidation–reduction gradient pattern in living, uninjured *Paramecium multimicronucleatum*, *Frontonia* sp., and *Spirostomum teres* (not stained by neutral red), and similar patterns are shown to be present in several other ciliate protozoans. Directions of progress in development and change of these color patterns are described as "posteropetal" or "anteropetal."

2. Except for modifications under extreme conditions, as noted below, rate of oxidation and reduction of indicators normally decreases posteropetally in the ectoplasm of *Paramecium* and *Frontonia*. There is also evidence indicating presence of a somewhat variable entoplasmic gradient. In *Spirostomum* a similar gradient is distinct in entoplasm as well as in ectoplasm; but in a varying percentage of individuals a short anteropetal gradient appears in the posterior region, probably in association with the frequent backward locomotion.

3. Under irritating conditions locomotion may become almost or quite continuously backward for several seconds or longer in all three forms; and the indophenol reaction and oxidation of reduced dyes are entirely or almost entirely anteropetal instead of posteropetal, suggesting increase of oxidase activity posteriorly with reversal of locomotion. This is most conspicuous in the very rapid backward locomotion of *Paramecium*.

4. In low oxygen, indophenol reaction or staining may appear only posteriorly with anteropetal gradient or may be

limited to the posterior entoplasm, or with Janus green red may appear anteriorly, in consequence of differential reduction of indicators at more anterior levels as rapidly as they enter. In very low concentrations of indophenol reagents or dyes, without decrease of oxygen, similar reversals of pattern apparently result from posteropetal alteration of destruction of indicators, which is not reversible and not prevented by high-oxygen content of the solution.

5. Intracellular oxidation in dye solutions completely reduced by sodium hydrosulphite is catalyzed by an oxidase, may occur within a few seconds or much less rapidly, and may progress posteropetally or anteropetally, according to conditions subject to experimental control.

6. Alterations of gradient pattern in the reconstitution associated with fission, particularly appearance of the new anterior end in the posterior member of the fission pair, are rendered visible by intracellular oxidation and reduction of the indicators. Even after separation, the rate of oxidation-reduction is slightly less in the posterior than in the anterior member of the pair.

7. A slight posteropetal staining gradient appears in the earlier stages of staining by oxidized dyes but may become indistinguishable as staining progresses.

8. All indicators used are photodynamically active in oxidized form. No conclusive evidence that they are active when completely reduced and colorless has been obtained. Posteropetal intracellular decoloration of oxidized dye by reduction or destruction, or both, is more rapid in light than in darkness and occurs without decrease of oxygen in the solution. Janus green is reduced directly to colorless in light.

9. Slight increase of pH accelerates the indophenol reaction and increases the differential in *Paramecium*, but above a certain pH the reaction is retarded. Increase of pH accelerates staining and increases the differential in the staining gradient; with neutral red, only very slight effect has been observed. Decrease of pH to neutrality or slightly below retards differentially indophenol reaction and staining by the four dyes, with decrease, obliteration, or reversal in direction of the color gradient. The similar modifications of indophenol reaction and staining by change of pH and other evidence suggest that "vital" staining may perhaps be correlated in some way with oxidase activity.

10. Neutral red, being an acid-alkali, as well as an oxidation-reduction indicator, can be used to indicate certain changes in intracellular pH. *Paramecium* in neutral red and nontoxic concentrations of KOH stains the same color as controls. In neutral red with KCN the intracellular color undergoes gradual change posteropetally toward alkaline. These data suggest that KCN enters the *Paramecium* cell in molecular form and decomposes into KOH and HCN within the protoplasm.

11. The patterns of indophenol reaction and staining in *Paramecium* in the presence of KCN are resultants of two factors: increased pH and decreased oxidase activity. With exposure to KCN m/500–m/100 and indophenol reagents or dye at the same time, reaction and staining are accelerated in the anterior ectoplasm; and at more posterior levels the reaction is retarded and dyes, except Janus green, are more or less reduced posteropetally without any experimental oxygen decrease. With exposure to KCN m/200 or m/100 for 10–20 minutes before addition of indophenol reagents or dyes, the indophenol reaction may be al-

most completely inhibited and dyes reduced almost or quite as rapidly as they enter because decrease of oxidase activity interferes with the indophenol reaction and permits dye reduction by decreasing utilization of oxygen present.

12. The most striking visible evidence of decrease of oxidase activity in *Paramecium* by cyanide appears in the very great retardation, after exposure to KCN, of intracellular oxidation in dye solutions reduced by hydrosulphite. If excess of hydrosulphite is avoided, oxidation in controls may be complete in a few seconds to a minute or two. In the same concentration of dye and hydrosulphite there may be very little oxidation after 5–15 minutes in animals previously exposed to KCN m/200 or m/100 for 15–20 minutes.

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RELATIONS BETWEEN THE REPRODUCTIVE CYCLE AND THE PITUITARY GLAND IN THE SNAKE THAMNOPHIS RADIX

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WHILE studies of the seasonal changes in the reproductive cycle of the cold-blooded vertebrates appear in an ever increasing number, few studies have appeared which correlate these changes in both sexes with variations in the hypophysis. The physiological interrelationship of the hypophysis with the gonads is well established in the vertebrates (see Marshall, 1936; Van Dyke, 1939; Severinghaus, 1939; and Moore, 1939, for summaries and bibliographies).

Cyclical changes in the hypophysis are best interpreted in relation to the behavior of organs which show marked seasonal variations, such as the gonads. Among the cold-blooded vertebrates, correlative changes have been reported in the fishes (Bock, 1928; Matthews, 1936), amphibians (Zahl, 1937; Aplington, 1942), and reptiles (Altland, 1939; Poris, 1941).

The male and female garter snakes chosen for this correlative study present many curious problems regarding pituitary control. Marked changes in the male gonads occur later than those occurring in the female. The sperm are produced during the summer months, while the ovocytes mature earlier. The sperm are stored in the ductus deferens during the fall and winter and are the effectual male germ cells the following spring. Copulation is not coincidental with fertilization and may occur long before ovocytes are mature. The female is ovoviviparous, with an approximate period of gestation of 9 weeks, during, and for a period after which, corpora lutea are present in the ovary. The corpora lutea in snakes have been shown to protect pregnancy (Fraenkel *et al.*, 1940; Clausen, 1940). Differential growth of ovocytes is present. Similarly, the pituitary state of the snake in hibernation, when the body temperature is considerably lower and all activities are minimal, is of interest.

Moore (1939) has pointed out the reciprocal action of gonadal-pituitary relations in warm-blooded animals. It is not unlikely that similar relations exist in the reptiles. Snakes have been shown to possess three sex hormones—estrone, progesterone, and an androgen (Valle and Valle, 1943). The effects of hypophysectomy and gonadotropins on the gonads of reptiles have been described.

In this study an attempt has been made to correlate the normal seasonal reproductive and pituitary cyclical changes in both the male and female garter snake,

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with the view of establishing relationships and a basis for physiological experiments.

MATERIALS AND METHODS

Only adult garter snakes, *Thamnophis radix* (Baird and Girard), were used for the seasonal studies. The male garter snake (20–51 gm.) is usually smaller than the female (42–164 gm.) and can be recognized externally by a thicker and longer tail. The female has an abrupt narrowing of the tail dorsoventrally, immediately posterior to the cloacal aperture. This difference is due to the presence of inverted hemipenes in the cloacal area of the male. In the male a probe can be passed into the sac formed by an inverted hemipenis (Schaefer, 1934). Sexual dimorphism occurs in the tail of the garter snake (Burt, 1928; Ruthven, 1908).

The animals used in this study were collected in a limited area of several square miles at the northern city limits of Chicago during the years 1937–38 and 1940–42. In all instances autopsy was performed within 1–3 days after collection, in order to avoid any changes under laboratory conditions. Hibernating specimens were removed periodically from an outdoor, escape-proof, wire-mesh cage ($7 \times 4 \times 4\frac{1}{2}$ feet), filled with leaves, or from a small indoor cage kept in a dark, cold-room (40° F.). A side door into the outdoor cage kept in a window pit permitted easy removal of the snakes with a minimum of disturbance to the environment. Usually the snakes were aggregated in the central part of the leaf pile. The top of the cage was covered with an oilcloth, to prevent undue wetting of the leaves. The skin of the snakes was moist or wet during hibernation, and the animals exhibited very sluggish movement, even when the outside temperature was below zero. It is of interest that snakes in the cold-room were often found in the water kept in the cage.

For the reproductive cycle 101 males and 111 females were used throughout a period of 2 years. The pituitaries were studied from 35 males and 67 females autopsied throughout the 1-year period, and distributed as shown in Table 4 (p. 318). A total of 36 experimental animals were kept in a greenhouse.

Weights and measurements of the gonads were taken on the fresh organs. Bouin's and Zenker's fixatives, the paraffin method, and Mallory's triple, Delafield's, and Heidenhain's hematoxylin stains were employed on the gonads. The pituitary glands, with a part of the brain and floor of the skull, were placed in Dawson's fixative for 4 hours, washed, and run up to 70 per cent alcohol and iodine, at which time the hardened pituitary was dissected out. They were stained lightly with eosin in order to locate the gland in paraffin. Serial sections were cut at 5 μ and stained with Mallory's Azan. Cell counts were made under oil immersion and with a 10× ocular, using a Zeiss ocular crossline micrometer (5 mm. square). With a Bausch and Lomb microscope this gave a field of 6,400 μ^2. For each animal all the cells in the field were counted in two sections of the pituitary, and the average used as the recorded count.

ANATOMY AND HISTOLOGY OF THE PITUITARY

A. ANATOMY

The hypophysis cerebri of an average mature garter snake gave maximum measurements of 780 μ in length, 918 μ in width, and 535 μ in height in the prepared section, although pituitaries may be larger or smaller than this, depending on the size of the snake. It consists of the pars anterior, intermedia, and nervosa (Pl. I, Figs. 1–4). No evidence of a pars

tuberalis is found in the adult. The vascular connective tissue from the anterior tip of the pars anterior penetrates and radiates in the thickened floor of the infundibulum (Figs. 1 and 3). This is the pars terminalis, which serves to attach the pars anterior. The anatomy of snake pituitaries has been considered by Siler (1936) and Hartmann (1944) and reviewed for reptiles by Charipper (1937). These observations on the pituitary of *T. radix* are presented as additional material to that given by Siler (1936) for the same species. Observations on the pituitary of *Thamnophis sirtalis* by Hartmann (1944) agree with those of Siler. Morphological and cytological studies of lizard pituitaries have also been made (Poris and Charipper, 1938; Altland, 1939). Poris and Charipper (1938) find similar divisions of the hypophysis in the lizard *Anolis carolinensis* except that a pars terminalis is absent.

Unlike the symmetrically arranged gland in other reptiles, the hypophysis of the snake usually has the bulk of the ventrally situated pars anterior displaced to the right and the pars nervosa to the left. However, occasionally in dissection the reverse relations have been noted, as well as a completely ventral and almost bilaterally symmetrical arrangement.

The residual lumen of Rathke's pouch is not the cleft between the pars anterior and posterior, which has never been found to contain colloid, but may be represented in part by the almost obliterated space of the pars intermedia, which often contains a "colloid" (Figs. 2 and 4) and the epithelial cysts.

The pars intermedia invests the pars nervosa closely and penetrates between the separate finger-like posterior projections of the pars nervosa (Figs. 1–4). The intermedia surrounds the nervosa ventrally, laterally, and posteriorly and thins out anteriorly on the dorsal surface (Fig. 2). It varies in thickness from a double layer of columnar cells to dense regions where this layer is folded or corded. The surrounding connective tissue, containing vascular channels, is carried in with the folds. No vascularity is ever present between the double layer of cells. The pars intermedia at its posterior end narrows into a "stalk," which continues into the pars anterior (Figs. 2 and 4), not unlike the situation in the lizard (Poris and Charipper, 1938). The pars anterior turns ventrally; laterally, it becomes larger and then extends anteriorly, where it attaches to the infundibular floor by the pars terminalis.

The pars nervosa is smaller in volume than the pars anterior, while the pars intermedia is the smallest. The infundibular cavity (Fig. 2) does not extend into the digitate processes of the nervosa, as seen in the lizard (Poris and Charipper, 1938). Not all digitate processes are of the same length or diameter (Figs. 1 and 2). Their number varies in different pituitaries (cf. Figs. 2 and 4). The infundibular cavity is continuous with that of the third ventricle.

B. EPITHELIAL CYSTS

Ciliated, partially ciliated, and nonciliated cysts of the pars anterior of the snake pituitary are quite common. They occur singly in 17 out of 75 females and in 6 out of 35 males examined. Four of the females possess two cysts. Cyst sizes vary from $24 \times 20\ \mu$ to $90 \times 65\ \mu$. The epithelium is quite variable in the different cysts. It may contain combinations of ciliated cuboidal epithelium, acidophiles, small and giant ($16 \times 12\ \mu$) basophiles, and stratified squamous epithelium. The cavity occasionally contains colloid, but usually it contains cellular debris in various stages of disorganization.

C. HISTOLOGY

The acidophiles, basophiles, and chromophobes of the pars anterior show zonation (Figs. 1 and 4). The acidophiles occupy the greater part of the anterior half of the gland and then become mixed with basophiles and chromophobes in a narrow transition zone (Fig. 3). The chromophobe region most often shows a corded arrangement of cells (Fig. 4). The acidophiles constitute the largest proportion of cells; basophiles, the least.

The acidophilic cells are of two types, which, for convenience, are referred to as "red" and "pink" acidophiles. Those in the acidophilic area are large ($12 \times 16\ \mu$), very irregular in shape, and often massed so that cell membranes are indistinguishable, heavily granular, and deep-red staining (Pl. II, Figs. 5*A* and 5*B*). Those in the chromophobe area (Fig. 1) that appear to be derived from the chromophobes are smaller ($8 \times 8\ \mu$) and more regular in shape (Fig. 6). The cytoplasm either appears as a ring around the 4–6 μ vesicular nucleus or as a mass bordering the sinusoid. The cytoplasm contains fine granules, which are light-pink staining. In *T. sirtalis* the latter have been designated as orange cells, since the fine granules stain yellow to orange (Hartmann, 1944).

It is readily apparent that acidophiles extend over a larger area of the pars anterior during the period of activity of the animal, whereas during hibernation the area is less extensive. The peculiar morphology of the pars anterior does not allow a more precise comparison of the extent of these areas. Similarly, their numbers are greater during the period of activity, as evidenced by the massing of cells, while during hibernation they are sparser and more individual.

Attempts to trace numerical changes in the acidophiles are made difficult by the massing of coarsely granular cells, which obscure not only cell membranes but also nuclei.

Throughout the year, acidophiles containing coarse granules can be seen, whereas the finely granular ones appear only during the active period of the animal (cf. areas in Figs. 1 and 3). The coarse granules are either sparsely distributed in the cytoplasm (Fig. 5*B*) or, as in the majority, so dense that they coalesce, giving a finely vacuolated appearance to the cytoplasm (Fig. 5*A*). Such finely "vacuolated" cells may show pycnotic nuclei. In some cells a few fine vacuoles attain a diameter of 1 μ. Several acidophiles have been seen with larger vacuoles.

The basophiles present two distinct types of cells, probably not unrelated. Numerous small basophiles ($6 \times 8\ \mu$) with deep-blue-staining granules, which may be so dense as to appear coalesced, are present (Fig. 7*A*). This imparts a fine vacuolar appearance to the cytoplasm. Larger vacuoles are also found in the basophiles. Nests of small basophiles may possess pycnotic nuclei (Fig. 7*B*).

Larger basophiles, fewer in number and ranging from 8×8 to $14 \times 14\ \mu$ in size, possess a light-blue staining, sparsely granular cytoplasm and large 6 μ vesicular nuclei (Fig. 8). These show variability in staining—sometimes almost a colorless condition. Vacuolization, pycnotic nuclei, and apparent dispersion of the cytoplasm are described in both sexes in the cyclic studies.

Chromophobes are small and stain poorly; their cell membranes are indistinct; very little cytoplasm is present; and usually the cells are quite crowded, as evidenced by their closely packed 3 μ nuclei (Fig. 9). Those in the chromophobe area sometimes stain a light lavender, not unlike cells of the pars intermedia.

Vascularization is quite pronounced in

the pars anterior. Blood cells are present in the sinusoids of most pituitaries (Table 4).

The elongated cells of the pars intermedia are arranged linearly in a double row, with the nucleated end of the cell centrad and the cytoplasmic end peripherad, the latter bordering the vascularized surface. The peripherad surfaces bound the pars nervosa and pars anterior. In thicker regions of the pars intermedia the covering vascular connective tissue is carried inward by folding. No vascular channels are ever found between the centrad ends of cells. Where the pars intermedia continues into the pars anterior by a narrowed "stalk," the vascularity of the former continues into the sinusoids of the latter.

Oxyphilic and basophilic granules in the same cells of the pars intermedia, bordering the cleft and basophilic granules in those cells adjacent to the pars nervosa, have been described (Siler, 1936). When Mallory's Azan was used, the greatest variability in staining reaction of these cells was encountered throughout the year in both sexes. The cytoplasm usually stained lavender to a deeper purple, was acidophilic and occasionally basophilic, or stained differentially, as described by Siler.

Variously sized clear-orange-staining globules, present in the "residual" lumen of the majority of the animals throughout the year, may be degeneration products derived from the nuclei of the intermedia cells, which show similar staining reactions (Figs. 2 and 4). Normal nuclei are vesicular and large ($6 \times 4\ \mu$). Vacuolization of the centrad ends of the cells is pronounced in some animals.

Occasionally cells appear to be free in the lumen, but they may be the sectioned ends of cells which have become isolated. However, one pituitary possessed several typical pars anterior basophiles; another, several acidophiles in the "residual" lumen.

SEASONAL CHANGES IN THE ADULT MALE

The male garter snake exhibited marked seasonal changes in weight and volume of the testes, associated with a single complete wave of spermatogenic activity during the summer. For this study a total of 101 males for the years 1937–38 and 1941–42, distributed as shown in Table 1, were used. The right

TABLE 1

SEASONAL TESTIS VOLUME AND WEIGHT CHANGES

Month	1937–38		1941–42			
	No. of Animals	Av. Vol.* (Cu. Mm.)	No. of Animals	Av. Vol. (Cu. Mm.)	No. of Animals	Av. Wt. (Mg.)
Apr.	5	165	3	219	3	179
May	5	243	5	216	5	116
June	3	252	4	243	4	163
July	8	476	3	558	3	366
Aug.	8	820	4	654	6	491
Sept.	7	724	8	489	4	386
Oct.	9	815	1	597	1	439
Nov.	5	204	.....	.....	1	386
Dec.	2	151	3	266	.....	.....
Jan.	1	175	2	146	.....	.....
Feb.	1	75	3	110	3	124
Mar.	8	186	.....	.....	3	114
Apr.	.....	.....	3	114	3	92
Total	62	.....	39	.....	38	.....

* Calculated for the ellipsoidal testis from measurements on the fresh gland.

ellipsoidal testis of the snake is usually larger and is located more anteriorly in the body. The mesial surface of the testis is bordered by an inconspicuous epididymis.

A. MACROSCOPIC CHANGES IN THE TESTES

The volume of the testes for two seasons, as shown in Chart I, parallel each other closely. The difference in maximum volume of the 1937–38 series was attributed to larger animals used. The weight changes paralleled the volume changes. Testes of the snake were maximal in weight and volume from July to the end of October; minimal from De-

cember to May. In May they began increasing and in November decreasing. The maximum volume was 990 per cent and 500 per cent greater than the minimal volume, while the maximum weight was 430 per cent greater.

The slight but consistent temporary drop in weight and volume of the maximum-sized testes may be due to an emptying of the seminiferous tubules of the sperm, mature at that time, as well as of "fluid." The seasonal decrease in weight and volume occurred during the latter half of November, just as the animals entered hibernation, and was followed by a tapering-off during early hibernation. Testes were minimal in size when the snake emerged from hibernation in March.

In May the testes were grayish white and flaccid, becoming firmer, with an increase in size, by July. Increased vascularity imparted a coral hue to the testes. When maximal in size, they were firm and white, with regression flaccidity increased and vascularity decreased.

Similar striking changes for other reptiles have been described, but only two species of turtles coincided in time relations: namely, maximal-sized testes in the summer, regression in fall, and minimal size in April and May, upon emergence from hibernation (Hansen, 1938; Risley, 1938).

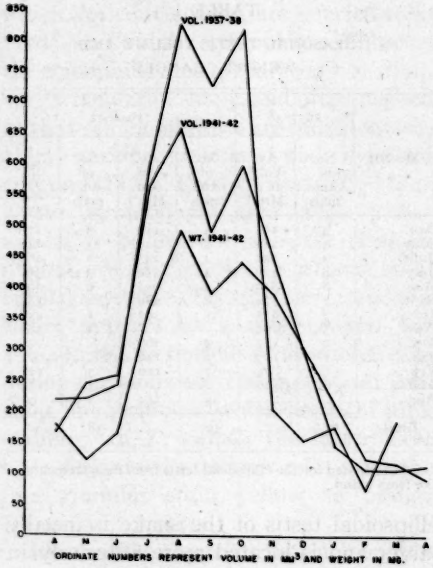
In two species of male lizard, maximal volume and weight changes of seventeen times and three times that of the minimal have been given; however, the maximum appeared earlier in the season, during May and June (Blount, 1929; Turner, 1935).

In three other species of lizards, nearly maximum- or maximal-sized testes were attained late in the season (August and early fall), were maintained throughout hibernation, and regressed in June (Woodbury, 1938; Altland, 1941; Reynolds, 1943).

Other groups of lizards fall in late-spring or early-fall categories (Kehl, 1935). These gross seasonal changes, it will be seen later, are associated not only with spermatogenesis but also with the time of breeding and sperm storage.

CHART I

VOLUME AND WEIGHT OF TESTES OF *Thamnophis* THROUGHOUT THE YEAR



B. SPERMATOGENESIS AND SPERM STORAGE

The spermatogenic cycle of the garter snake, as revealed by the study of the testes throughout the year, occurred as one complete wave during the period of activity of the animal. In hibernation no spermatogenic divisions take place.

The garter snakes in this area entered hibernation by November 1, although on unusually warm days an occasional snake would venture forth after this date as well as before March 1, about which time they began to emerge from hibernation.

Upon the snakes' entering hibernation the testes were not completely regressed in size. The seminiferous tubules were much collapsed, and the majority of the lumina were devoid of sperm. The germinal epithelium, however, still contained patches of elongated sperm heads and spermatids (Pl. III, Fig. 18). The picture during 4 months of hibernation showed a slow progressive destruction of these unevacuated and incompletely differentiated cells (Fig. 19). In the minimal-sized testes the tubules were one-half the diameter of the maximal-sized testes. In these reduced tubules the lumen was obliterated by debris derived from the germinal epithelium. This debris showed progressive signs of disintegration from pycnotic nuclei, multinucleated masses, and packets of sperm to a more homogeneous vacuolated granular mass. The germinal epithelium, much contracted by the shrinkage of the tubule, concentrated the nuclei of the Sertoli and the spermatogonial cells into a dense ring. There was no spermatogenic activity, only regressive changes, in the garter snake during hibernation.

Upon emergence from hibernation the lumina became clear. In April spermatogonia were dividing and primary spermatocytes appeared (Fig. 16). In June and July there was an intense spermatogenic proliferation, associated with the marked increase in size of the testis. By the end of July a few sperm tails were projecting into the lumen. Spermatids were abundant in the germinal epithelium. Only a few secondary spermatocytes were seen. Spermiogenesis was marked in August, and numerous sperm tails projected into the lumen (Fig. 17). The early stages of spermatogenesis were completed. Late in September sperm appeared free in the lumen of the seminiferous tubules, and they passed into the epididymis by the first part of October. The passage of sperm was associated with the temporary decrease in the size of the testis. The remaining spermatids undergo spermiogenesis during the latter part of October. Rapid evacuation of most of the remaining sperm from the testis occurred just prior to entering hibernation.

Hansen (1938) found a correlation of spermatogenesis with seasonal size changes in the turtle *Terrapene carolina*, similar in time relations to that in the garter snake. In another turtle, *Sternotherus odoratus*, Risley (1938) described a single wave of spermatogenesis, with a further emptying of the seminiferous tubules in the spring. In the turtles the sperm were stored in the epididymis and used in fall or spring breeding.

Fall matings were reported for *T. radix* (Coues and Yarrow, 1878) and for *T. sirtalis* (Blanchard and Blanchard, 1941*b;* Blanchard, 1943). Trapido (1940) summarized evidence for fall mating in other species of snakes. As motile sperm can be secured from the ductus deferens of *T. radix* during hibernation, it is not unlikely that fall breeding is possible. Copulation, however, occurred most commonly after emergence from hibernation, from March until June, and may be repeated several times. Insemination may precede ovulation for varying periods, and consequently fertilization is delayed. The percentage of motile sperm in the ductus deferens markedly decreased in July and disappeared before the new sperm entered the ductus deferens. Therefore, only sperm produced during the preceding season are effective in fertilization.

Lizards with maximal-sized testes in the fall and throughout hibernation underwent an incomplete wave of spermatogenesis, with arrested activity during hibernation and the completion of spermiogenesis in the spring (Woodbury,

1938; Altland, 1941). Those lizards with minimal-sized testes during hibernation underwent spermatogenesis and achieved maximal size in the spring, utilizing the sperm in spring or early summer breeding (Blount, 1929; Turner, 1935; Clausen and Poris, 1937).

C. CHANGES IN THE INTERSTITIAL CELLS

Interstitial cells are present in the testes of the garter snake. They have been described for the lizards, snakes, and turtles (see Blount, 1929, and Herlant, 1933). The amount of interstitial tissue varied in the different groups. Herlant (1933) reported scattered little masses in the lizards and more voluminous masses in the ophidians and chelonians. Altland (1941) found no interstitial cells in *Sceloporus u. undulatus*. Mazzetti (1911) reported them as abundant in a hibernating snake. In the hibernating garter snake the interstitial cells occurred in compact nests in the intertubular spaces. Relative to the regressed state of the seminiferous tubules, they appeared abundant, whereas in maximal-sized testes the cells were dispersed and appeared relatively less voluminous.

The interstitial cells in the lizards were best developed when the secondary sex characters were at their maximal activity in May (Herlant, 1933; Regamey, 1935). In the adder the interstitial cells did not appear to vary (Herlant, 1933), nor did Risley (1938) observe any changes in the musk turtle. Blount's (1929) quantitative study of seasonal variation in number and morphology of interstitial cells and volume of interstitial tissue demonstrated an increase in volume and a decrease in number concomitant with spermatogenic activity.

Seasonal observations on the interstitial cells of the garter snake revealed evidences of secretory activity and variation in size and staining.

In the spring the cells were large (16 × 18 μ) and contained eccentrically placed, spherical, vesicular nuclei (8 μ); they occurred in compact nests; their cell membranes were distinct and presented a polyhedral pattern (Fig. 16). The cytoplasm was light-staining; the fine granules formed a delicate alveolar protoplasmic pattern. Only an occasional cell showed an irregularly shaped, smaller (6 μ), denser nucleus and vacuolated cytoplasm.

In July and August the cells were more dispersed, although a few nests still appeared. Some cells, with irregular, darker nuclei, had very light vacuolated cytoplasm and, because of the indistinct cell membranes, appeared to be continuous with a similar light-staining intertubular substance showing vacuolation. It appeared that these cells were actively secreting (Fig. 17). Smaller cells (10 × 12 μ), with a distinct cell membrane, denser cytoplasm, and an eccentrically placed (6 μ) nucleus, and the spherical (8 μ) cells with 5 μ vesicular nuclei may be either postsecretory or new cells.

In October, just before entering hibernation, the large interstitial cells were again crowded in nests. No intertubular substance was present. The most striking condition was the presence of enormous interstitial cells (14 × 26 μ) with 8 μ, vesicular nuclei. The cytoplasm was homogeneous or vacuolated (arrow, Fig. 18). Other cells averaged 10 × 12μ and possessed a vesicular nucelus.

During hibernation many of the large cells (14 × 16 μ) were vacuolated but had a distinct cell membrane and an irregular small nucleus (arrow, Fig. 19). The remaining cells measured 10 × 12 μ, and possessed a 6 μ nucleus. The cytoplasm in the latter was denser. In some in-

stances cell membranes were indistinct where the cells occurred in groups.

In the garter snake large interstitial cells are present in the spring; during the summer they are small and are dispersed in an intertubular substance; in the late fall they attain their greatest size; in hibernation the large cells are vacuolated, while the smaller cells occur in groups and possess an indistinct cell membrane and an irregular nucleus.

The lizard *Phrynosoma* had different time relations in its spermatogenic activity and breeding. Breeding immediately followed the production of sperm. Blount (1929) correlated the greatest size of the interstitial cells with the height of the breeding season.

Breeding in the garter snake does not immediately follow the production of sperm, as in the lizard *Phrynosoma*. However, there is a possibility of fall mating in the garter snake, at which time interstitial cells are large, some attaining enormous size. Interstitial cells in the garter snake are also large in the spring, when breeding is pronounced.

D. CYCLICAL CHANGES IN THE PITUITARY OF THE MALE

Thirty-five adult males, distributed throughout the year 1941–42 (Table 4), were used for the study of the seasonal cell changes in the pituitary. Although the pituitaries were from animals sacrificed weekly, the counts represented in Table 4 are monthly averages. As described earlier, two counts of acidophiles, basophiles, and chromophobes were made on each animal.

Certain inherent difficulties are present in making a quantitative analysis of the snake pituitary. Because of the peculiar asymmetrical, teardrop shape of the pars anterior, it is not possible to make a strip count which gives the relative proportions of the areas occupied by chromophiles and chromophobes. However, qualitative observations have been made on serial sections, and these will be added. A second factor that is readily apparent, when using a given area, is the variation caused by the distended or collapsed state of the sinusoids and by differences in cell size. Both large and small, acidophiles and basophiles, are combined in the counts. A separation of each chromophile into two types, on the basis of size, would imply that the cells are not related. Also, cells of intermediate size would be difficult to place. Attention is also called to the fact that, whereas a second peak in number of basophiles is present during hibernation, these cells are predominantly small in size; their increased number does not mean that physiological activity is present. Because the areas selected are in a transition zone where the basophiles are present, the counts of acidophiles and chromophobes are not a true indication of their actual abundance. Qualitative changes of all the cells will be considered; numerical changes, only for the basophiles.

In the pars anterior two types of acidophiles were described earlier. The coarsely granular acidophiles are present throughout the year in the acidophilic zone. They are abundant and closely packed in this zone during the spring, summer, and fall, and sparsely distributed during hibernation. Throughout the year the majority of these acidophiles show deep-red-staining granules, so packed as to appear coalesced and "vacuolated" (Fig. 5*A*); the remaining ones have fewer deep-red granules, which are sparsely distributed in the cytoplasm and are seen chiefly in the transition zone from spring until fall (Fig. 5*B*). The latter acidophiles may be in a process of degranulation. Orange-staining nuclei are present in some

acidophiles during the summer and may be indicative of an altered physiological state.

The finely granular, pink-staining acidophiles are most abundant and pronounced in the chromophobe area during the spring; they become fainter in the summer and fall, decreasing in abundance until in hibernation the chromophobe area is almost pure chromophobes. These finely granular acidophiles are small in size; they contain little cytoplasm; and the nuclei are centrally placed or acentric and stand out clearly. They are not unlike the chromophobes in morphology and are located in a zone which is chromophobic during a part of the year. Their origin from the latter appears certain. Between the coarse acidophiles, which are sparse during hibernation, are chromophobes. These chromophobes must be the source of coarse acidophiles that fill this region during the active period. The two types of acidophiles do not appear to be stages of secretory activity of the same cell but do appear to arise independently from chromophobes. However, in *T. sirtalis*, Hartmann (1944) describes carmine (red) and orange (pink) acidophiles as morphologically different and states that some carmine cells arise directly from chromophobes, whereas others are derived by transformation from orange cells.

The coarsely granular acidophiles are least abundant in hibernation, and the finely granular acidophiles are most pronounced in spring. Both types of acidophiles are active in producing secretions when climatic and food conditions are optimal for growth (see discussion for association of acidophiles with growth hormone on p. 322).

The basophiles are generally considered in the literature to be the source of gonadotropic substances (Severinghaus, 1939). Seasonal changes in the number of basophiles, as represented in Chart II, reveal two peaks of abundance. The higher and more significant peak occurred during the period of testicular activity, while the second, lower peak occurred during hibernation, when testicular activity was absent.

In the spring the large light-blue basophiles are most abundant. They show the most pronounced cytological change—a change which is undoubtedly associated with the elaboration of gonadotropic substances. The granules are fine and are dispersed in the cytoplasm. The staining reaction of the cells decreases until they are almost colorless. Cell membranes become progressively less distinct. Vacuolization appears in the cytoplasm, and many nuclei become orange in color and irregular in shape. Only a few small deep-blue basophiles are present at this time.

In *T. sirtalis* during periods of decrease in basophile percentage many of the cells stain less blue than the rest, and pycnosis of the nucleus may or may not be present. The cells contain extremely fine blue granules in a colorless cytoplasm with a quite centrally situated nucleus and a prominent nuclear membrane (Hartmann, 1944).

The variability in staining response of the basophiles encountered by three workers on garter-snake pituitaries, in spite of attempts at a uniform technique throughout the year, is sufficient reason to believe that the condition of this cell varies.

In the summer, only a few light-blue basophiles, with either scattered granules or vacuoles, are present. Small bodies, 3 μ in size, which occur in nests, appear to be the end stage of light-blue, vacuolated basophiles which have given up their secretion (Fig. 7*B*). Only a faint-blue, irregular cytoplasmic border surrounds a much shriveled nucleus which

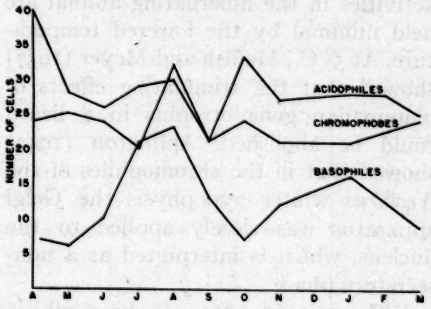
may be temporarily pycnotic. Whether these bodies degenerate, form chromophobes, or regenerate new basophiles is difficult to ascertain. The small deep-blue basophiles are appearing at this same time in ever increasing numbers. Their nuclei are quite dense and spherical and in some cases they are orange and irregular. The coarse granules of the cytoplasm are closely packed and appear coalesced. This gives a finely "vacuolar" appearance to the cytoplasm (Fig. 7*A*). Occasionally, larger vacuoles do appear in these cells. When judged by their shape, size, and amount of cytoplasm, the deep-blue basophiles seem to be derived from chromophobes, although the nuclear condition might lead one to believe that they arise from the expended basophiles by regeneration. That both phenomena may occur in the pituitary has been shown by the study of mitotic activity, which gives rise to a new supply of chromophobes (Hunt, 1942), the degeneration of cells (Kirkman, 1937), and regeneration (suggested by Aplington, 1942).

In the fall the light basophiles are no longer apparent; only small deep-blue cells with a homogeneous granular cytoplasm are present. Nuclei are clear and vesicular. These cells gradually increase in size and in number. They are the dominant basophile of the hibernating snake. A few, slightly larger basophiles, with a homogeneous light-blue cytoplasm which are present during hibernation, apparently arose from the deep-blue cells and will become the large light-blue basophiles seen in the spring pituitary.

Of the two seasonal peaks in basophile number represented in Chart II, only the summer peak can be correlated with maximum gonadotropic activity. At the time of emergence from hibernation and during the spring there was a progressive decrease in the number of basophiles. Cytologically, the basophiles exhibited marked secretory activity in the spring, when testicular activity was stimulated. During the height of testicular activity in the summer, small deep-blue basophiles increased in number and in extent. Whether the fine "vacuoles" and occasional larger vacuole in the small basophiles represent activity is not certain. Light-blue basophiles were present during the summer but decreased toward the end of the spermatogenic period.

CHART II

SEASONAL CELLULAR CHANGES IN THE MALE PITUITARY, 1941–42



When the testes attained their maximal size in the summer, the basophiles were three times as numerous as during the spring. Physiologically, the pituitary is active gonadotropically during this period, as demonstrated by experiments in Section E, below. The decrease in basophile number and extent in September and October was associated with a rapid decline of spermatogenic proliferation and a temporary decrease in the size of the testes. Along with the decline in number of basophiles, sperm appeared free in the lumen of the seminiferous tubules in September and in the ductus deferens in October. It may be that the release of sperm from the testis is induced by gonadotropic substances, just as ovulation may be induced in the female.

When the basophiles are minimal in number in late October (the testes have completed a secondary wave of spermioteleosis, and interstitial cells have attained an enormous size), a rapid decrease in size of the testes occurs. As climatic and food conditions become less favorable, the animals enter hibernation. The basophile number slowly increases upon entering hibernation, does not attain the height of the first peak, and does not show the cytological changes associated with secretion. Spermatogenic activity is absent in the testis, and interstitial cells are vacuolated. Cellular activities in the hibernating animal are held minimal by the lowered temperature. At 5° C., Mellish and Meyer (1937) showed that the stimulating effects of mammalian gonadotropins in a lizard could be abolished. Aplington (1942) showed that in the chromophiles of the *Necturus* winter hypophysis the Golgi apparatus was closely applied to the nucleus, which is interpreted as a nonsecretory phase.

Differential counts of chromophiles and chromophobes in 22 male garter snakes (*T. sirtalis*) revealed two peaks in basophile numbers also (Hartmann, 1944). These peaks occur in April and September. The reproductive cycle of this species, distributed around Ithaca, New York, is not known; but Hartmann indicates that the increased basophile number may possibly be correlated with increased size of the testis and with spermatogenic activity at these periods.

E. HYPOPHYSECTOMY AND REPLACEMENT EFFECTS ON THE TESTES

Twenty garter snakes were hypophysectomized on June 30 and July 9 (Table 2), to determine to what extent the pituitary gland, which showed increasing numbers of deep-blue basophiles and relatively few light-blue basophiles, was secreting a gonadotropic substance. At this time the testes were markedly increasing in size (Chart I), and spermatogenesis was quite intense.

The snakes were anesthetized by a subcutaneous injection of sodium amytal, supplemented by ether. The actual operation is relatively simple because the easily located basisphenoid projection containing the pituitary is approached directly and is not obscured by the palatine bones. Bleeding was minimal or absent; healing rapid; and infection absent. Hellbaum (1936) presents details of the operation as described by Schaefer (unpublished).

Pituitaries have been successfully removed in other cold-blooded vertebrates. Matthews (1939) found that *Fundulus* males that were hypophysectomized in fall and kept until the next breeding season (spring) failed to show typical testis enlargement, while males hypophysectomized in March or April, when testes are enlarged, showed a reduction in size below that of the controls, and, in addition, spermatogenesis was affected. Similar regressive changes have been reported in another teleost, *Gobius pagnellus* (Vivien, 1938, 1939). Marked retrogression in size and histological disorganization of the testes in *Amblystoma tigrinum* (Burns and Buyse, 1932) and marked involution in *Triton cristatus* (Woronzowa and Blacher, 1930) have been reported. Hypophysectomized cold-blooded vertebrates showed marked changes in the testes and a gonad relationship similar to that established in higher vertebrates.

Male garter snakes hypophysectomized on July 9 and autopsied 9 and 16 days later (controls for replacement experiments) showed only a slight difference in weight of the testes, when compared with the normal cycle (Chart I). Histologically, after 9 days the germinal epithelium un-

derwent a marked sloughing of cells, leaving the epithelium as a thin ring of cells. The sloughed cells completely filled the lumen of the seminiferous tubules. A few tubules showed pycnotic cells and macrophages (Fig. 10). Interstitial cells are clumped and small. Sixteen days after hypophysectomy the tubules are much shrunken. Pycnosis, dissolution, and vacuolization are pronounced (Fig. 12). Interstitial cells are small and compacted, and they possess color and flaccid. Histologically, the solid seminiferous tubules (63 μ in diameter) showed a germinal epithelium disrupted, vacuolated, and containing cellular debris and pycnotic nuclei, or a single row of spermatogonia and Sertoli cells (Fig. 14). No active spermatogenic divisions were present. Interstitial cells occurred grouped either with irregular nuclei containing dense chromatin, with indistinct cell membranes and little cytoplasm, or individually (measuring 10 $\times$

TABLE 2

HYPOPHYSECTOMY AND REPLACEMENT EFFECTS ON THE TESTES

No. of Animals	Treatment (1942)			Average Animal Weight (Gm.)	Testes	
	Hypophysectomy	Autopsy	Treatment		Average Weight (Mg.)	Average Volume (Cu. Mm.)
3	June 30	Aug. 12	None	24	40	46
3	June 30	Sept. 3	None	28	36	39.9
3	Intact	Sept. 3	None (*control*)	30	393	406
5	July 9	July 18	10 male pituitary homeoimplants each	30.6	380	.....
2	July 9	July 18	Control	28.5	185	.....
5	July 9	July 25	Total each 350 I.U. Gonadin (equine gonadotropin)	28	421	.....
2	July 9	July 25	Control	36	227	.....

irregular nuclei and vacuolated cytoplasm.

Hypophysectomized males showed over a prolonged period (operated on June 30 and autopsied 44 and 66 days later) a marked reduction in the testis weight and volume (Table 2). The normal control testis size was ten times greater than that of the experimental animal. When the atrophy during this period is compared with the size changes in the normal seasonal cycle (Chart I), a 50 per cent reduction below that which occurs during hibernation (testes normally minimal in size) is found.

Macroscopically, the testes of the hypophysectomized snakes were tan in

12 μ; nuclei, 3–5 μ) with vacuolated cytoplasm, densely chromatic, irregularly shaped nuclei, and an occasional vesicular nucleus. No sperm were present in the ductus deferens.

The coral-colored testes of the normal controls, in marked contrast to the experimentals, showed large distended lumina in the seminiferous tubules (150 μ) and a thick germinal epithelium with abundant normal divisions in the later stages of spermatogenesis (Fig. 15). The spermatids and elongating sperm heads were most numerous. Sperm were present and free in the lumen. The testis of the controls corresponded in size and phase of spermatogenesis to that seen

in the normal cycle. The intertubular connective tissue showed a pronounced vascularization and wide areas of a finely granular, light-staining colloid in which the interstitial cells were mostly scattered (Fig. 11). The interstitial cells were large (12 × 16 μ), with eccentrically placed vesicular nuclei and finely alveolar, light-staining cytoplasm. Cells measuring 12 × 14 μ had a densely chromatic nucleus and a heavily vacuolated, light-staining cytoplasm which was not distinguishable from the surrounding vacuolated substance of the intertubular space. Smaller cells (8 × 10 μ) had a distinct cell membrane. An occasional spherical interstitial cell (7 μ) with dense, heavier-staining cytoplasm contained an irregularly shaped densely chromatic nucleus. It appeared that the interstitial cells in phases of secretory activity contributed to the abundant colloidal material surrounding the cells. The interstitial cells of the controls were like those in the normal cycle on September 3. When the interstitial cells of the hypophysectomized animals are contrasted with those of the controls, far less evidence for secretory activity was found to be present in the former. The cells were smaller in size, contained less cytoplasm, and possessed denser, irregular nuclei.

In hypophysectomized *T. radix* and *T. sirtalis*, Schaefer (1933) observed "small and flaccid testes with degenerating spermatocytes in the atretic seminiferous tubules and the size of the interstitial cells less than normal." His observations were made during the winter months, when the testes are normally either regressing or minimal in size and inactive.

Removal of the pituitary gland in July (at a stage when small deep-blue basophiles are increasing in number and degranulating light-blue basophiles are present in relatively fewer numbers) caused the failure of testicular activity. Animals hypophysectomized over a prolonged period during the summer showed atrophy and aplasia of the testes. The withering of the gland following hypophysectomy to a level below that which occurs in hibernation would indicate that even in the reduced inactive testes of hibernating animals there is hypophyseal influence on the testes.

July male pituitaries were used for implantation to determine their gonadotropic potency. Fresh glands were macerated in distilled water and injected intraperitoneally. Each of five hypophysectomized males received a total of ten glands in four injections. The weight of the testis after 9 days was twice that of the controls (Table 2). Compared with the normal cyclical changes (Chart I), when the testes rapidly increase in size, the homeo-implants produced not only the normal 50 per cent increase in weight during this period but also an added stimulation of 50 per cent. Spermatogenesis was abundant and normal, with differentiation of sperm (Fig. 11). Interstitial cells were mostly intermediate in size, and the cytoplasm dense. Only a few larger cells (17 × 16 μ) were present (cf. with Fig. 10, described above). The gonadotropic potency of July male pituitaries was sufficient not only to maintain testicular activity and produce the normal increase in testis weight during this period but also, in the amount administered, to stimulate a further increase. Schaefer (1933), in winter snakes, reported that subcutaneous implantations of a single hypophysis every other day for a period of 30 days elicited germ-cell production, enlarged tubules, and increased size of interstitial cells.

Another group of five hypophysectomized males was daily injected intraperitoneally with equine gonadotro-

pin (Table 2). Each received a total of 350 I.U. and was autopsied 16 days later. The weight of the testis was practically doubled, and spermatogenesis was abundant. Interstitial cells were all greatly enlarged, and many of them were vacuolated. (Cf. Fig. 13 with Fig. 12 [hypophysectomized control described above] and Fig. 11.) This stimulation, like the homeo-implant stimulation, was similarly in excess of that which occurs normally in the seasonal cycle.

The effectiveness of mammalian pituitary extracts in stimulating reptilian gonads has been demonstrated. This is considered later in the discussion.

only slightly higher in number than that in the control, extended over a wider area. When compared with the normal basophile cycle, the number was found to be low at the time of castration. It is significant that, whether testes are present or absent, the basophiles show a threefold increase during this phase of the seasonal reproductive cycle. This same phenomenon may account for the slight increase in percentage of basophiles reported by Poris (1941) for the castrate male lizard *Anolis*. In *Triturus viridescens* 3½-month castrates showed an increase in basophiles two to three times the normal (Copeland, 1943).

TABLE 3

CASTRATION EFFECT ON THE PITUITARY OF THE MALE

No. of Animals	Treatment	Av. Weight of Animals (Gm.)	Av. Weight of Testes upon Removal (Mg.)	Av. Pituitary Cell Counts (Acidophiles-Basophiles-Chromophobes-Erythrocytes)
2.....	Castrated 5/2/42; autopsy, 8/12/42	21	70	27–20–23–3
2.....	Castrated 5/2/42; autopsy, 9/4/42	35	97	19–25–23–9
3.....	Normal control; autopsy, 9/4/42	30	427	23–20–19–9

F. EFFECT OF CASTRATION ON THE PITUITARY IN THE ADULT MALE

Castration of four males was performed on May 2, 1942, at a time when the basophile count in the normal cycle was at its lowest (Chart II). Two animals were autopsied after 103 days; and two others after 124 days, when three controls were sacrificed. Not only did the weights of the testes of the castrates at the time of removal (av., 83 mg.) and of those of the normal controls at the time of autopsy (av., 427 mg.) (Table 3) agree with the weights of the testes in the normal cycle, but the histological picture at those phases of the cycle was also the same.

One hundred and twenty-four days after castration the basophiles, although

Cytologically, pituitaries of castrates and controls showed a predominance of small deep-blue basophiles with coarsely granular cytoplasm, either dense, appearing "vacuolated," or scattered. The basophiles of the castrate extended over a greater area and contained a larger percentage of orange nuclei (cf. Figs. 20 and 21). Only a few light-blue basophiles (described in the normal cycle) contained scattered coarse granules, and occasionally small vacuoles were present. Small bodies described in the normal cycle and believed to be expended basophiles were present also in the castrate. Coarsely granular and finely granular acidophiles were present in both castrate and control; however, the finely granular pink acidophiles were still quite numerous and

distinct in the castrate, while in the controls only a few faint ones were present. No acidophilic mass in the basophiles, as reported in 4½-month castrate lizards (Poris, 1941), was observed in the snake.

SEASONAL CHANGES IN THE ADULT FEMALE

The reproductive cycle of the female, in contrast to that of the male, involved production of mature eggs and their utilization during the same season. This ovoviviparous species gave birth to young after a gestation period of 9 weeks.

The elongated saclike ovaries varied in length from 2.5 to 10.5 cm. The right ovary was always more anterior than the left and 0.8–1.6 cm. longer. The ovocytes were linearly arranged, giving the gonad a beaded appearance. Ovocytes of several sizes were distinguished; smaller ones were translucent, and larger ones opaque-yellow. As yolk accumulated, the ovocytes enlarged. At their maximum size the ovocytes measured 1.6 × 1.1 cm. and were orange-yellow in color.

Upon ovulation a corpus luteum formed, persisted throughout pregnancy, and regressed very slowly after parturition. The luteal tissue arose from the granulosa layer (Rahn, 1939) and has been described for other reptiles (see Fraenkel *et al.*, 1940; Boyd, 1940). Post-pregnancy females could be identified definitely up to and during early hibernation by the still vascularized, unregressed oviducts, as well as by the corpora lutea. In the spring the corpora lutea had regressed to small orange specks and were not distinguishable from those discernible from former pregnancies. The number of corpora lutea scars was not suitable for certain identification of more than one pregnancy, since the garter snake has a variable number of young per brood. Mature virgin females could be readily identified not only by the absence of corpora lutea scars but also by the presence of an imperforate membrane, where the paired Müllerian ducts join the urodaeum.

A. COPULATION

The female is inseminated in spring, prior to ovulation. One pair was observed in the act of copulation in the field on May 1. The pair remained in coitus ½ hour after being collected. In the field, on April 27, and in the laboratory, on April 29, courtship was observed. An account of courtship behavior and mating in the garter snake has been described by Davis (1936) and the Blanchards (1942); in other species, by Noble (1937). Sperm have been found in the oviducts of spring females but not in hibernating females. (See the section on the male, Part B, for a discussion of fall copulation.) The sperm are held until fertilization, which occurs in late May or early June, at the time of ovulation. Rahn (1940) reported motile sperm in the washings of the oviducts of *T. s. sirtalis* 1 month before fertilization, which occurs during June and early July. In the copperheads, sperm live 11 days or longer in the female (Gloyd, 1934). Isolated oviparous species of snakes have remarkable records of sperm viability in their reproductive tract, as evidenced by their laying of successive clutches of fertile eggs. Sperm in the female are capable of fertilization in the African night adder over a period of 81 days (Woodward, 1933), in the Malayan water snake over a period of 1½ years (Kopstein, 1938), and in a Central American leptodeiran over a period of at least 5 years (Haines, 1940).

B. PERIOD OF GESTATION

The period of gestation in the garter snake was approximately 9 weeks. In 1941 the earliest ovulation was observed on May 31, and the latest date of no

ovulation was on June 21 (Chart III). Whether the phenomenon occurs in the early or late parts of this period is probably dependent on the temperature (Blanchard and Blanchard, 1941*a*). The earliest parturition occurred on August 2, and the last female to be pregnant was observed on August 23. Most of the females had given birth, however, the first week in August. There was a 3 weeks' variation at either end of the period of gestation. Assuming that the earliest ovulation produces the earliest brood, the period of gestation would be 9 weeks. Variations in gestation periods for other species of snakes have been found (refer to Clausen, 1936, for summary), as well as variations in the incubation period of oviparous snakes. As shown for this ovoviviparous species, the gestation period cannot be dated from the time of copulation. The unduly long gestation periods reported need re-examination.

C. EFFECT OF HIBERNATING CONDITIONS ON THE GESTATION PERIOD

Six females in the latter half of their gestation period were placed under hibernating conditions (dark, cold-room, 40° F.) on July 23, 1942, and removed to room temperature after 70 days, to determine the effect of temperature on the period development of the embryo. None gave birth to fully developed young during this period. One died upon removal, October 21; two on November 12; a fourth on November 23; and a fifth on April 23, 1943. Upon autopsy the retained young were found to be in various stages of resorption and showed that development had been arrested. Half the amount of yolk present in embryos in the latter half of their developmental period was still present. The sixth pregnant female aborted (five times between January 30 and March 15, 1943) a total of 9 dehydrated, dead, half-grown embryos and was still alive on April 26. The embryos undoubtedly died during the period of hibernation, as evidenced by their degree of development.

D. NUMBER OF YOUNG IN THE GARTER SNAKE AND CORPORA LUTEA

The number of developing young in the garter snake was quite variable, ranging in 1937–38 from 9 to 22, in seven females, averaging 15.4 young per female. In 1941–42 the developing young ranged from 6 to 26 in twenty-six females, averaging 12.4 young per female. In a large (37 inches) *T. s. sirtalis* a brood of 73 young was reported by Wallace (1938). Usually the number of young is much fewer. Blanchard and Blanchard (1942) found in their extensive studies on *T. s. sirtalis* that the number of young varied from 6 (the first brood of a 3-year-old female) to 51. In eight gravid *T. s. sirtalis*, Burt (1928) found a range of from 12 to 32 young, or an average number of 16.9 young; and in five *Thamnophis sauritans sauritans*, a range of from 4 to 8, or an average number of 6 young. For the latter subspecies Ruthven (1908) found 12 young. Kopstein (1938) reported similar ranges in seven other ovoviviparous species of snakes.

An analysis of the distribution of young in the oviducts showed an average of 8 in the right and 4.4 in the shorter left. In these same females the corpora lutea average 8.4 in the right ovary and 6.1 in the left ovary, which indicated that not all ovulated ovocytes developed. Corroboration was found in several instances of undeveloped, dehydrated ova in the oviducts. An analysis as to whether ovulated ovocytes entered their respective oviducts revealed, in four cases, that migration occurred from the left side, anteriorly to the right oviduct. Similar observations were made in the turtle (Agassiz, 1857).

Where fewer ova than corpora lutea occurred, there is the possibility that the ova were lost. That contractions in either direction, causing expulsion of the contents of the oviducts, can occur during gestation is believed to be indicated in the following two cases. A coelomic pregnancy, autopsied 2 months after birth should have occurred, showed young in stages of resorption. This may have been caused by a reversed contraction sometime during development. Another female undoubtedly aborted her young during mid-pregnancy, as evidenced by heavily vascularized, collapsed oviducts, well-developed corpora lutea in the ovaries, and a high basophile count.

E. SEASONAL CHANGES IN THE OVARIAN CYCLE

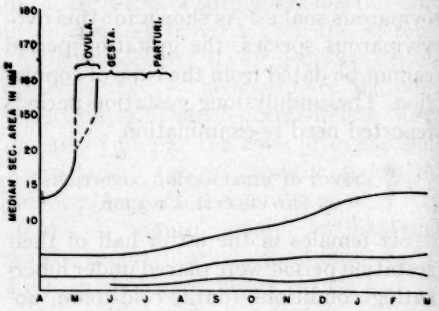
The ovaries and reproductive tract showed seasonal changes (Cieslak, 1938). The area of a median longitudinal section of the ovocytes offered a convenient index for size changes. These changes in 111 females for the years 1937–38 and 1941–42 are represented by the average mean curves of Chart III.

Three distinguishable size groups of ovocytes were present in the mature female, which could be measured macroscopically. The largest size group in May rapidly increased in size from 13 sq. mm. to 140–226 sq. mm. The maximal size variation appeared to be associated with the total number of ovocytes. Where large numbers were present, the mature yolk-laden ova were smaller. Ovulation of this group occurred on the last day in May and during the first part of June. Corpora lutea developed and were large during pregnancy, regressing slowly after parturition. It is believed that the tiny orange specks that are present during hibernation in mature females having their oviducal membranes ruptured represent corpus luteum scars. From the numbers of these in some females it appeared that they had accumulated from former pregnancies. Large virgin females with unruptured membranes never showed these corpus luteum scars.

During the growth of the first group of ovocytes there was little change in the size of the second and third groups. At the end of June, after the females had ovulated and most of them were in mid-pregnancy, a new group of tiny ovocytes, slightly less than 1 sq. mm., made its appearance, restoring the three size groups

CHART III

CHANGES IN THE THREE GROUPS OF OVOCYTES OF 111 *Thamnophis* THROUGHOUT 1937–38 AND 1941–42



in the ovary. There was a very slight growth of the new first and second groups in July and August. During the early part of September all three groups grew slowly; and they continued to grow through the first half of the hibernation period, at which time growth leveled off, so that, upon emergence from hibernation the following year, the three groups attained the same size categories seen the previous year. The number of ovocytes in each of these three categories showed the same variation in numbers as the broods.

In some animals, such as the mouse, the germinal epithelium becomes active at each normal oestrus period, forming

cyclic proliferations of germ cells and follicle cells (Allen, 1923). Woodbury (1938) described part growth of lizard ova in fall, no change in winter, and rapid enlargement in spring, when ovulation occurred. Four well-marked sets of different-sized ovocytes have been described in the ovary of *Chrysemys picta* (Agassiz, 1857). In the fish *Cottus bairdii* a portion of the ovocytes formed during the first season matures for the first spawning, which takes place at the age of 2 years. The remainder form a reserve supply, which is increased each year by ovocytes from dormant oögonia (Hann, 1927).

F. SEASONAL CHANGES IN THE FEMALE PITUITARY

In considering the cyclical pituitary changes in the female, it was necessary to consider the sixty-seven mature females in four categories: (1) nonpregnant females (April–June), (2) pregnant females, (3) postpregnant females, and (4) mature virgin females. The determination of these categories was quite definite, according to criteria described earlier, in Part E, on the ovarian cycle. The females were distributed throughout the year, as shown in Table 4. Cell counts of the pituitary in Table 4, plotted in Chart IV, necessarily overlap, because of the 3-week leeway in the period of gestation.

Cyclical changes in the coarsely granular acidophiles, present throughout the year, were similar to those in the male. These were large, irregular in shape, and "vacuolated" throughout the year. During hibernation they were less concentrated in the acidophile zone. In the spring the granules in some of these cells were scattered, and in the fall this condition was more pronounced. The cells with scattered granules may be degranulating and returning to a chromophobe state, which would then account for their sparser distribution during hibernation. The finely granular pink acidophiles undergo a more marked seasonal change. In the nonpregnant females and the pregnant females they are abundant in the chromophobe area. In the postpregnant females in hibernation they are absent.

The dominant basophiles in the nonpregnant females in April and June are of the large light-blue type. The cytoplasm is sparsely granular and contains numerous tiny vacuoles or a few larger ones (Fig. 8). Cell membranes are indistinct, and the cytoplasm becomes almost colorless. Those cells lining the sinusoids appear disrupted, as if discharging the light-blue colloid in the sinusoid with which they are continuous. Orange nuclei are also seen. The light-blue basophiles showing cytological evidence of secretion occur also in pregnant females but appear fewer in proportion to the deep-blue basophiles in the latter half of pregnancy. In postpregnant females in the fall they are still present and active but fewer in number. In early hibernation a few smaller light-blue basophiles with scattered granules are preparatory for the more numerous, larger light-blue basophiles with tiny vacuoles, which appear in late hibernation.

The deep-blue basophiles are not apparent in the spring but appear in early pregnancy and become very numerous in late pregnancy. They are present in the postpregnant females in fall, predominate in early hibernation, form two-thirds of the cells in late hibernation, and give rise to the light-blue basophiles in spring. During pregnancy and postpregnancy in the fall, the presence of coarse granules in these small deep-blue cells (which appear to be coalesced, giving a "vacuolated" appearance), occasional larger vacuoles, and variable amounts

TABLE 4

DISTRIBUTION OF ANIMALS AND MONTHLY AVERAGE OF PITUITARY CELL COUNTS

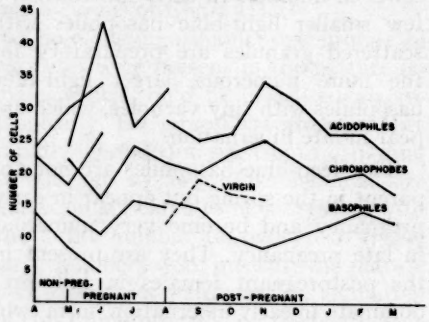
Month (1941–42)	No. of Females (Acidophiles-Basophiles-Chromophobes-Erythrocytes)*			No. of Males (Average Count)
	Nonpregnant	Pregnant	Postpregnant	
April	3 (24–14–22–6)			2 (40–7–24–0)
May	11 (30–8–18–6)	2 (24–14–23–9)		4 (28–6–25–6)
June	4 (34–4–27–6)	8 (43–10–16–4)		4 (26–10–24–5)
July		10 (27–16–24–5)		3 (29–21–20–1)
Aug.	2 (30–12–19–12)†	3 (33–14–21–6)	5 (28–15–21–6)	4 (30–23–32–2)
Sept.	1 (21–19–26–4)†		8 (25–14–20–5)	5 (21–13–21–0)
Oct.	1 (20–17–19–17)†		2 (28–10–23–5)	3 (33–7–24–2)
Nov.	2 (23–16–16–9)†		1 (34–8–25–4)	3 (27–12–21–14)
Dec.				
Jan.			2 (26–12–19–14)	2 (28–16–21–10)
Feb.			1 (26–14–20–21)	2 (28–12–21–15)
Mar.			1 (17–12–17–12)	3 (25–9–24–8)
Total number.	24	23	20	35

* Numbers in parenthesis represent the order of cells and are the averages for the animals used that month.
† Cell counts in virgin females.

of cytoplasm and the fact that one-fourth of the cells have orange nuclei would indicate either transitional stages in light-blue basophile development or

CHART IV

SEASONAL CELL CHANGES IN THE FEMALE PITUITARY, 1941–42



secretory activity. Their increase in number and subsequent decrease may serve as a constant source of supply for the active, degranulating, and vacuolating light-blue basophiles which revert to chromophobes or degenerate.

The numbers of basophiles throughout the year reveal, as in the male, two maximal peaks (Chart IV): one during pregnancy and the other during hibernation. Upon emergence from hibernation a two-thirds decrease in number (marked secretory activity of light basophiles) is undoubtedly associated with the release of gonadotropic substances, which become effective the middle of May, when climatic and food conditions improve, and which produce a tremendous growth of the largest group of ovocytes (Chart III).

During early pregnancy, when the number of basophiles is maximal, a temporary drop in number may be correlated with the growth and macroscopic appearance of a new third group of ovocytes. Otherwise, little growth is apparent in the remaining ovocytes during the period of gestation, when basophiles are most abundant.

In the postpregnant female, as basophiles decrease in number during September and October, there is continued slow growth of the three macroscopic groups of ovocytes into early hibernation, when the stage of ovocyte develop-

ment found in the emerging females is attained. Evidence of secretory activity of light basophiles is present on into fall, when the deep-blue basophiles increase; these predominate during early hibernation and give rise to the large light-blue basophiles that dominate in the spring.

Corpora lutea present in the ovary during pregnancy and slowly regressing in September and October bear a relationship to the pituitary (Clausen, 1940) and may account for the sex difference in basophile number, which is lower in the female. Mature virgin females possessed a larger number of basophiles than the pregnant females (Chart IV). Their number was more nearly like that found in the male. The number of basophiles remained high in fall, when those of the postpregnant female decreased.

DISCUSSION

A correlative study of the reproductive cycle in the male and female garter snake (*Thamnophis radix*) and of the pituitary gland necessitates the delineation of phases of the former and quantitative and qualitative alterations in the latter. Gonad activity of the snake living in a temperate climate is characterized by morphological and physiological changes during the spring, summer, and fall and an abeyance of activity in hibernation. In contrast to the single annual rhythm in this snake, reptiles in regions where climatic conditions are fairly constant may show repeated internal rhythms. Oviparous Javanese snakes lay eggs at intervals throughout the year (Kopstein, 1938), and the New Hebrides lizard (*Lygosoma*) breeds all year round (Baker, 1929). The annual fluctuation in metabolism in garter snakes that is associated with environmental change permits the single internal rhythm of gonadal activity to occur. However, these events in the two sexes are not concurrent, as the male produces sperm the season prior to their use.

The testes of the garter snake undergo the most marked seasonal size changes in the summer, in this respect being like the turtle (Hansen, 1938; Risley, 1938). Lizards fall into two groups: those which attain maximal-sized testes in the spring, producing sperm for use in spring or early summer; and those which attain maximal-sized gonads in the fall, maintain during hibernation, and complete spermioteleosis in the spring (see Parts A and B in the section on the male for further discussion). The activity of the testes, which is most pronounced during the period of maximal size, shows the inception of spermatogenesis in May and probable secretory activity of the large interstitial cells, as evidenced by a smaller, denser nucleus and vacuolated cytoplasm. Similarly, when spermiogenesis is completed in the fall and the testes are regressing in size, interstitial cells attain their maximal size. This testicular activity during the spring and fall may be associated with the decreasing number of basophiles in the spring and fall.

The female possesses three groups of ovocytes. The largest group shows marked growth in May and early June but has a previous history of slow growth in former seasons. Upon ovulation and during subsequent gestation not only are corpora lutea present in the ovary but also the new third group of ovocytes appears in the ovary. Outside of the slight growth of this set of ovocytes to bring them into the macroscopic realm, there is little growth of the three groups during the remainder of the gestation period. Parturition in August is followed by regression of the corpora lutea and further growth of the three goups of ovocytes in the fall, when they attain the size seen in the spring. Even though the ovary shows most marked activity in the

spring, there is a slow growth present during early pregnancy and in the fall. As in the male, this gonadal activity may be associated with the two periods of basophile decrease.

Both sexes show gonadal activity from May until November. The marked changes in size concerned with the production of mature germ cells are not concurrent in the male and female. The production of sperm occurs after the female has ovulated, and under no circumstances could they be used in fertilization that season. The testes are minimal in size during hibernation and are not producing sperm. Sperm storage in the snake takes place in the ductus deferens, from where motile sperm can be secured over a period of 8 months. (In the turtle, sperm are stored in the epididymides, while lizards with maximal-sized testes in the fall do not complete spermiogenesis until spring.) Courtship and copulation may extend through the spring, when gonadal activity is in its incipient stages. Copulation may not involve insemination; nor is insemination immediately followed by fertilization, which occurs at ovulation the first part of June. Sperm storage occurs in the female from the time of insemination until fertilization. Consequently, the gestation period must be based on the time of ovulation and parturition data, rather than on mating dates. Difficulty in determining ovulation dates in a closely related species led the Blanchards (1941*a*) to speak of the "period of development" as "the time between the start in the egg of preparation for fertilization (which happens probably a month after mating) and the time of birth of young, without regard to either the point at which fertilization occurs or how long beforehand copulation takes place." In *T. sirtalis* the gestation period estimated from data presented by the Blanchards is approximately 71 days. In *T. radix* the gestation period is approximately 9 weeks.

Physiological activity of the gonad hormones in the garter snake is concerned with the preparation of the reproductive tract for the reception of mature germ cells and a possible reciprocal action on the pituitary gland. That an estrogen is produced in snakes has been demonstrated by the assay of extracts of the ovaries of 324 specimens of *Bothrops jararaca* and *Crotalus terrificus terrificus* by Valle and Valle (1943). These authors state that Porto (1941) found progestational substances in the corpora lutea of Crotalidae. Fraenkel *et al.* (1940), in their studies on viviparous snakes, have shown that deluteinization of the ovary stopped development of embryos and that they were never born alive. Clausen (1940) found that ovariotomy in viviparous snakes (*Natrix*, *Thamnophis*, and *Storeria*) in early and middle gestation caused the resorption of yolk or the abortion of young, while in late gestation normal young were produced. Assay for androgen from the snake testes gave positive effects (Valle and Valle, 1943). The effect of the sex hormones in reptiles on the sex characters has been demonstrated by ablation and substitution experiments. They will not be considered here except to point out that normal quantitative relation of the hormone to the sex characters and to the pituitary gland or the excretion of the sex hormone is not known.

Sex hormones, administered to reptiles, may cause reduction in gonad size. Injections or the implantation of pellets of estrogens into male lizards (*Anolis*. *Eumeces*, and *Sceloporus*) produced a reduction in the size of the testes (see Poris, 1941; Forbes, 1941); into the female *Anolis*, no significant effect on the ovary. Testosterone pellets implanted

into male *Sceloporus* caused a reduction in testicular volume and a stimulation of spermatogenesis (Forbes, 1941), whereas injections did not affect the testes (Gorbman, 1939). Variable effects reported in reptiles are probably due to differences in dosage, mode of administration, and the time of administration. Moore, in the rat, interpreted reduction in gonadal activity following administration of sex hormones as being operative through the pituitary gland.

Cytological study of male and female *Anolis* pituitary glands following estrogen injection during the winter revealed decreased staining capacity and vacuolization of basophiles. This was interpreted as an indication of degranulation and increased secretory activity. Distention of blood sinusoids occurs, and the accumulation of colloid-like material is suggested as representing gonadotropic or thyrotropic hormone (Poris, 1941). The author also interprets vacuolization of basophiles as a degeneration change due to overstimulation. Severinghaus (1939) interprets the degranulating cells with enlarged Golgi apparatus and mitochondria as producing secretion. The exhaustion of the pituitary would lead to a decreased gonadotropic potency and cause partial involution of the gonad (see Fevold, 1939).

Castration in *Anolis* over a period of 3 months (February–April) and sacrifice at intervals of 3 weeks to $4\frac{1}{2}$ months revealed no obvious histological changes in the pituitary, but the basophiles did increase slightly in number (Poris, 1941). In the experimental garter snake (castration at a low point in the normal basophile cycle and autopsy 124 days later) the number of basophiles increased to the same extent as the controls, which was a threefold increase from the initial level. The basophiles in the castrates, however, extended over a wider area. Cytologically, there was no apparent difference; both showed numerous small deep-blue basophiles and relatively few light-blue basophiles.

Little is known regarding the effect of other endocrine glands on the pituitary gland in reptiles except for the work of Siler (1936), who thyroidectomized eight *T. radix* in the fall and eight in the spring. The fall animals were kept at 70° F. and were autopsied from 90 to 101 days later. The spring animals kept at room temperature, which varied with the outdoor temperature, were autopsied from 9 to 66 days later. Changes in the cells of the anterior lobe as a result of thyroidectomy as presented by Siler (1936) are:

> (*a*) A transformation and enlargement of the chromophobe cells with an accumulation of basophilic granules to form, as a final stage, functional (?) beta cells. (*b*) Vacuolation of the beta cells with a loss of their fine granules to form a large non-granular cell, whose cytoplasm is chromophobic. (*c*) Degeneration of these transformed beta cells; the final form being a small cell with pycnotic nucleus and a very small rim of non-granular poorly staining cytoplasm. (*d*) A reduction in the number of alpha cells.

Similar changes were observed in the normal cyclical study of the pituitary gland, with normal fluctuation in temperature and thyroid function. Neither the sex of the animals nor the date of autopsy was given; nor was it stated whether gonadal function was initiated under winter laboratory conditions. In the lizard *Uromastix acanthinurus* an increase in the number of oxyphiles following thyroidectomy was reported by Viguier (1911). Changes due to thyroidectomy have been ascribed to all three types of cells by different investigators. Irrespective of the cell concerned, it is of interest to note here something of the mechanism of control of secretion, as determined by physiological studies of the potency of the pituitary, which

may have a parallel in gonadal-pituitary relations. No difference in physiological potency of normal, castrate, and thyroidectomized rat pituitary on the guinea-pig thyroid activation was observed (Hohlweg and Junkmann, 1933); but thyroxin-treated rat pituitaries had their thyrotropic effect decreased.

Abundant evidence is available for the control of testis function (Moore, 1939) and ovarian function (Van Dyke, 1936, 1939; Fevold, 1939; Smith, 1939) by two gonadotropic substances produced by the pituitary gland. A seasonal study of the cell changes in the pituitary gland of the snake, with a single annual reproductive cycle, offers certain advantages, which a short-cycle form like the rat does not offer, in that a given cytological condition may not be associated with the reproductive state at that time but the condition a day or two later. In the snake two types of acidophiles, which appear to arise independently from chromophobes, include large, coarsely granular, deep-red acidophiles and smaller, finely granular, pink-staining acidophiles. The former are present throughout the year in the acidophile area. They are abundant and more closely packed during the period of the animals' activity, whereas in hibernation chromophobe cells are present between them. In the spring and fall, cells with the granules dispersed have been interpreted as being in the process of degranulation.

The finely granular, pink-staining acidophiles appear in the chromophobe area and are apparently derived from those chromophobes, resembling them in size and shape. In the male they become fainter in the late summer and fall; in the female, in the fall. In both sexes they are chromophobic in the winter. Poris (1941) observed the pars anterior of *Anolis* as being deeply acidophilic in late summer, fall, and winter and faintly acidophilic in the spring.

Cytological activity is evident in the basophiles, which are of two types; but they are believed to be phases in the activity of a single cell. Small, coarsely granular, deep-blue basophiles in the transition zone of the pars anterior are present in the summer, fall, and during hibernation. In the spring, upon emergence from hibernation, they practically all enlarge, degranulate, stain light blue (some nuclei stain orange and are irregular), vacuolate, disrupt, and become colorless, giving rise to small bodies with dense nuclei and a thin rim of irregular cytoplasm (summer). Whether these bodies degenerate or regenerate is not known. Large light-blue basophiles dominate in the spring, and, relative to the deep-blue basophiles, are fewer in number during the summer and fall in the female, not apparent in the male in fall, and practically absent in hibernation except for a few intermediate-sized light-blue cells.

Siler (1936) in *T. radix* and Hartmann (1944) in *T. sirtalis* recognized the types of acidophiles which have been considered here as two types, because of their difference in position and behavior. The basophiles have been considered in two categories, for convenience in relating their state to reproductive changes. Types of chromophiles in *Rana pipiens* and *Necturus maculosus* have also been described on the basis of granules and staining reactions (Zahl, 1937; Aplington, 1942).

The acidophiles have been associated with growth. Hereditary dwarfism in the mouse is associated with the absence of acidophiles, which can be restored with fresh anterior-lobe implants. Acromegaly and gigantism are associated with pituitary tumors, which are acidophilic. Pre-

dominantly acidophilic tissue of the ox pituitary has been shown to cause growth in tadpoles, whereas no such effect is observed when predominantly basophilic tissue is administered (Smith and Smith, 1923). The growth hormone from the ox pituitary has recently been isolated and found to cause a resumption of growth in hypophysectomized rats. In the dosage used, the preparation is free of other biologically active pituitary contaminants (Li and Evans, 1944).

The basophiles are generally considered in the literature to be the source of gonadotropic substance in the vertebrates (see Severinghaus, 1939, for review). Physiological investigation of the pituitary gland of reptiles concern effects of hypophysectomy, pituitary implantation, and gonadotropin injection.

Garter snakes, hypophysectomized for a short period when basophiles were increasing in number rapidly, showed a slight decrease in the weight of the testis and a sloughing-off of the germinal epithelium, indicating that the pituitary gland was actively producing a gonadotropic substance at this time. Longer periods (16, 44, and 66 days) produced a further regression of testicular size and a loss of testicular activity. Ten pituitary gland homeo-implants from July males into hypophysectomized males not only maintained normal spermatogenesis during a period of 9 days but also stimulated a 105 per cent increase in testicular weight. Schaefer (1933) hypophysectomized male garter snakes (*T. sirtalis* and *T. radix*) during the winter months and implanted a single hypophysis every other day for a period of 30 days; he reported the enlargement of tubules, the production of germ cells, and an increase in the size of the interstitial cells. The pituitary glands of winter animals under laboratory conditions may not be identical with those from animals in hibernation. However, these winter glands did produce stimulation, which indicates some storage of gonadotropic substance.

It is known that the pregnant female pituitary produces a gonadotropic substance which maintains the corpora lutea, for it has been shown that hypophysectomy, ovariectomy (Clausen, 1940), and deluteinization (Fraenkel *et al.*, 1940) stop development of young, producing abortion or resorption. Implantation of five homeoplastic hypophyses into a single snake (*Xenodon merremi*) induced ovulation in 6 days (Houssay, 1930); ovulation also occurred after five homeoplastic implants in *A. carolinensis* (Evans, 1935*a*).

Mammalian gonadotropins (Gonadin) injected into hypophysectomized garter snakes (during 16 days) maintained normal spermatogenesis, greatly stimulated the interstitial cells, and produced an 84 per cent increase in testis weight. Mammalian pituitary extracts failed to correct the increased respiratory quotient in the hypophysectomized snakes *Pituophis sayi sayi* and *Elaphe guttata* (Roth, 1942). In normal reptiles gonadotropins produce stimulation of the gonads of male *Eumeces laticeps* during the period of quiescence (Turner, 1935); of the male and female *A. carolinensis* (Evans, 1935*a*, *b*); of *Phrynosoma cornutum* (Mellish, 1936); and of immature male and female alligators (Forbes, 1937).

In the reptiles very few reports on cyclical changes in the pituitary gland are available. Altland (1939) found that only the female lizard (*Sceloporus u. undulatus*) had predominantly basophilic cords in May, when sexual activity was at its height. Poris (1941) reported an apparent increase in the number of faintly staining basophiles in *A. carolinensis* during the spring and early summer, when

gonads are most active. Hartmann (1944) found seasonal variation in the basophile number of *T. sirtalis* males.

A seasonal analysis of the basophile number in the male and female garter snakes (*T. radix*) shows two peaks—one during the period of maximal-sized testis and of pregnancy and the other during hibernation. They both have approximately the same seasonal onset and regression. The hibernation peaks are similar in numbers in the two sexes, and both show a predominance of small deep-blue basophiles and only a few intermediate-sized light-blue basophiles. This pituitary condition is interpreted as inactive in producing secretion; gonadal function is in abeyance during hibernation—in fact, all activities are at low ebb in hibernation. The peaks during the period of pregnancy and of maximal-sized testis differ in number in the two sexes. That in the male is higher than in the female and represents a sex difference. Even though the total number of basophiles is high and the majority of them are the small deep-blue basophiles, secretory activity is present during these periods in both sexes, as evidenced by the degranulation of the remaining light-blue basophiles.

Cytological evidence of the secreting basophile cells and evidence of the decrease in the number of basophiles, correlated with gonadal changes, are found in both sexes in the spring, when the ovaries show pronounced growth and testicular activity is initiated. Small deep-blue basophiles begin to increase and are most numerous during pregnancy and during maximal testis size. These are interpreted as a new supply of basophiles, which probably are not actively secreting but form a supply from which cells constantly pass into a secretory phase. That the male pituitary is producing a gonadotropic substance even though the small deep-blue basophiles are increasing in number in July is shown (1) by the removal of the pituitary gland, which causes cessation of testicular activity, and (2) by the implantation of July pituitary glands, which maintains and stimulates testicular activity. That the pregnant female basophile cells are producing a gonadotropic substance in the first half of pregnancy, when their number fluctuates, is believed to be indicated by the growth of the new third group of ovocytes.

In the female, following gestation, there is a slow decrease in basophile number as the supply of deep-blue basophiles is diminished by becoming large light-blue basophiles which degranulate. Corpora lutea during this period are regressing. The slow growth of the ovocytes at this time indicates the production of a gonadotropic substance by the pituitary gland. This second regression in basophile number is not so striking as the pronounced degranulation of basophiles in the spring decrease in basophile number. The basophile number of the mature virgin female is higher than that of the pregnant female and more nearly like the number found in the male. The cells are predominantly small deep-blue basophiles and have been interpreted as being inactive in producing gonadotropic substance. Their number remains high even after the postpregnant female basophile number regresses. No apparent growth of the ovary was observed in the virgin females.

In the male the decrease in basophile number occurs after the first spermiogenic wave. That gonadotropic substance is produced is indicated by the increase in interstitial cell size.

In both sexes, in fall and during hibernation, small deep-blue basophiles in-

crease in number and predominate. A few intermediate-sized light-blue basophiles appear arrested in activity, as are all processes in the animal. Increasingly and decreasingly favorable climatic and food conditions in spring and fall, respectively, undoubtedly play a role in the degree of production of gonadotropic substance by the basophiles. During the optimal period of gonadal activity, gonadotropic substance is being produced. Sex hormones are similarly being produced and utilized by the reproductive tract. The degree of this utilization may supply the variable for reciprocal action on the control of the secretory activity of the basophiles.

SUMMARY AND CONCLUSIONS

1. The pars anterior shows zonation of three types of cells: acidophiles, basophiles, and chromophobes. Two types of acidophiles are described. Basophiles are grouped into light-blue and deep-blue cells, for convenience. The female has fewer basophiles during the reproductive peak than the male. This constitutes a sex difference. However, the basophile number of the mature virgin female is more nearly like that found in the male. The pituitary of the castrate male after 124 days shows a threefold increase in basophiles, which is slightly greater than the increase in the normal male.

2. There are seasonal changes in the pituitary of both sexes: (*a*) Coarse acidophiles are present throughout the year; they are abundant during the period of activity and scattered during hibernation. (*b*) Pink acidophiles in the chromophobe area in the male are present in the spring, becoming fainter in the summer and fall; they are present in the nonpregnant female in the spring and the pregnant female and absent in both sexes during hibernation. (*c*) Deep-blue basophiles are present in the summer, fall, and during hibernation and in the spring give rise to the dominant large light-blue basophiles. (*d*) Light-blue basophiles, dominant in the spring, show extensive degranulation; they are relatively fewer during the summer and during hibernation.

3. In both sexes there are two peaks in the number of basophiles: (*a*) They have nearly the same seasonal onset and regression. Regression in the spring is correlated with marked gonadal activity; in the fall, with ovocyte growth and increase in interstitial cell size. (*b*) The hibernation peaks are similar and cannot be associated with gonadotropic activity. (*c*) The peaks during reproduction differ (sex difference), and secretory activity is present. (*d*) A slight fluctuation in basophile number during early pregnancy may be associated with the appearance of the new third group of ovocytes.

4. Animals hypophysectomized for a period of 9, 16, 44, and 66 days during the summer peak in basophile number showed a progressive atrophy and aplasia of the testis. July pituitary homeo-implants produced a 105 per cent increase in testis weight over a period of 9 days in hypophysectomized snakes. A total of 350 I.U. of equine gonadotropin over a period of 16 days increased testis weight 84 per cent in hypophysectomized snakes. In both groups spermatogenesis and interstitial cells were stimulated.

5. The maximal volume of the testis is from 500 to 990 per cent greater than the minimal volume, and the maximal weight is 430 per cent greater. Spermatogenesis occurs as a complete wave during the late spring and summer, with the most pronounced activity during June and July, which is associated with the maximal weight and volume of the testis.

Spermiogenesis is pronounced in August, with a secondary wave in late October. Sperm are stored in the ductus deferens during hibernation and are utilized the following spring. Interstitial cells show seasonal changes in size, staining, and cytology.

6. Three macroscopic groups of ovocytes show differential growth and ovulate in succeeding seasons. Corpora lutea persist during a 9-week gestation period, regress after parturition in August, and leave corpus luteum scars. The average number of young in 33 females is 13. Embryonic development ceases under hibernating conditions.

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PLATE I

(Magnification, 42×)

ABBREVIATIONS

P.I., pars intermedia
P.N., pars nervosa
P.T., pars terminalis
I.C., infundibular cavity
E.C., epithelial cyst
Co., "colloid" of pars intermedia
S, "stalk" of pars anterior
R, area of red acidophiles
P, area of pink acidophiles
B, transition area of basophiles
C, area of chromophobes

FIG. 1.—Sagittal section of a May pituitary gland, showing the acidophile, basophile, and chromophobe areas of the pars anterior, the digitate pars nervosa (*P.N.*), and investing pars intermedia (*P.I.*). The anterior portion of the pars anterior (*top*) is separated, owing to the asymmetry of the lobe. The edge of an epithelial cyst (*E.C.*) is visible. (No. 26B, female.)

FIG. 2.—Several sections parasagittally to Fig. 1, showing the further extent of the pink acidophile area, the "colloid" in the residual lumen, the infundibular cavity, and the varying lengths of radiating digitate processes of the pars nervosa.

FIG. 3.—Sagittal section of a January pituitary gland, showing a more extensive corded chromophobe area, owing to the absence of pink acidophiles, the dark masses of blood cells in the sinusoids, the narrow basophile area (*B*), and the vascular pars terminalis (*P.T.*). (No. 260B, female.)

FIG. 4.—Several sections parasagittally to Fig. 3, showing the attachment (*S*) of the chromophobe area (*C*) to the pars intermedia (*P.I.*). Note the "colloid" in the residual lumen. The vascularity of the chromophobe area extends onto the surface of the pars intermedia. Digitate processes are fewer in number.

PLATE II

(Scale: $\frac{3}{8}$ Inch Equals 8 μ)

FIG. 5*A*.—Three red acidophiles (12 × 16 μ), showing the irregular cell shape, the indistinct cell membranes, and the dense granules, which appear coalesced, giving a finely "vacuolar" appearance to the cytoplasm. Some vacuoles are 1 μ in diameter.

FIG. 5*B*.—Three red acidophiles lining a sinusoid, showing the large coarse granules scattered and packed in the cytoplasm.

FIG. 6.—Several pink acidophiles (8 × 8 μ), showing a regular, spherical shape with a ring of finely granular cytoplasm and a vesicular nucleus. These cells are present in the chromophobe area.

PLATE I

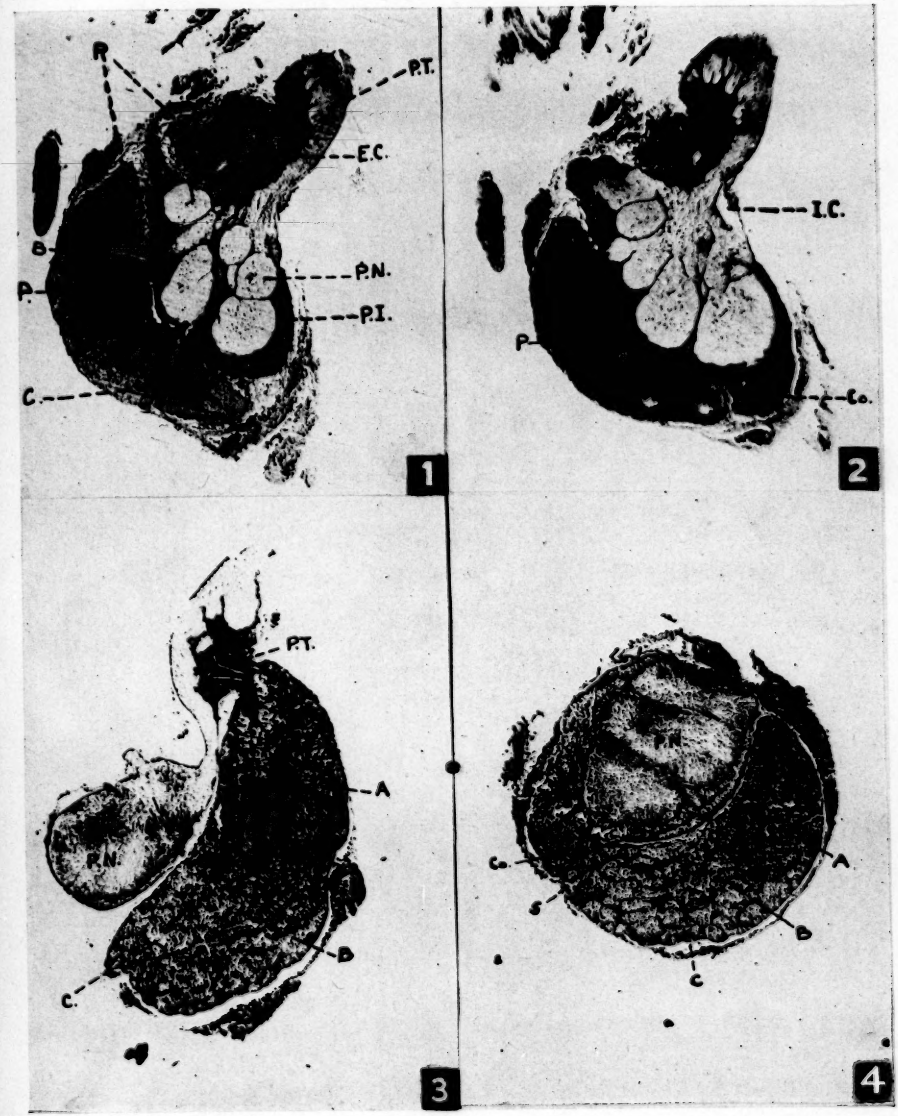

PLATE II

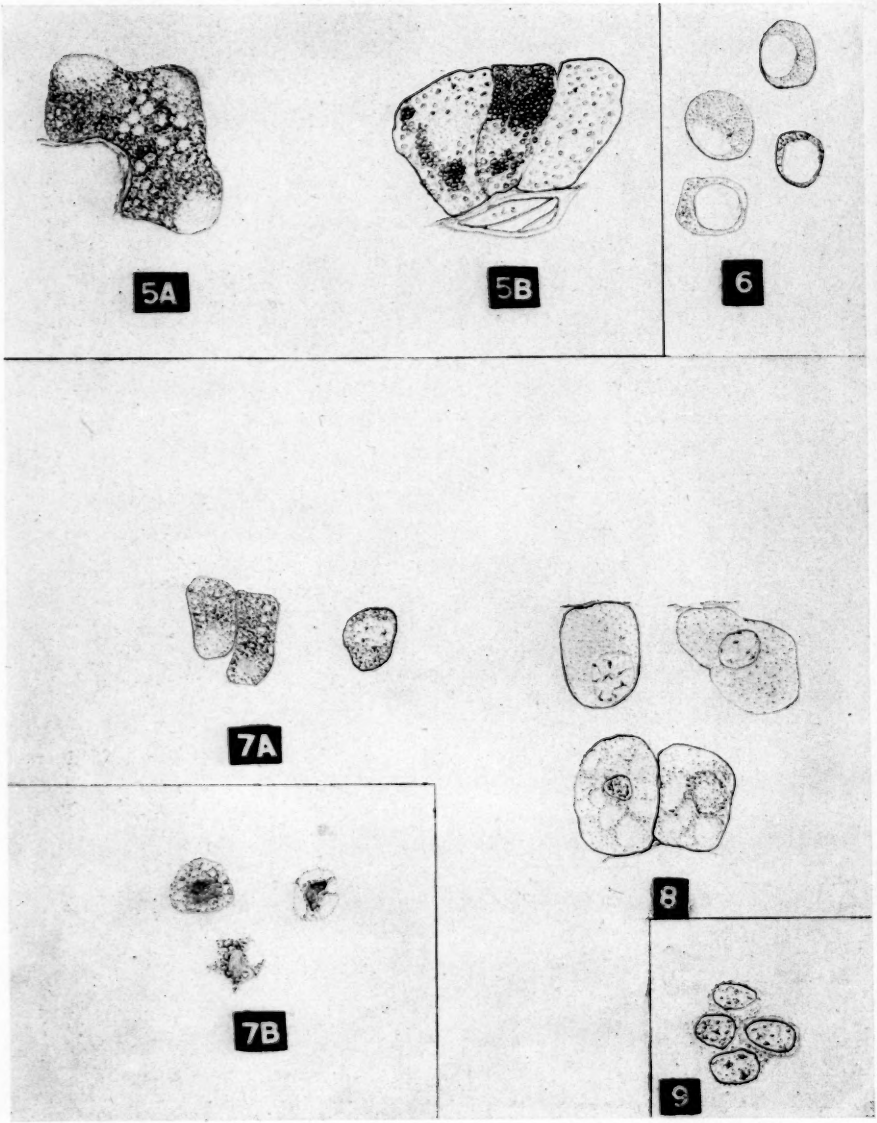

PLATE III

FIG. 7*A*.—Three small deep-blue basophiles (6 × 8 μ), showing the irregular shape of the cell. The dense granules appear coalesced, and one cell possesses a dense nucleus.

FIG. 7*B*.—Small vacuolated basophiles and basophile "bodies" with orange nuclei represent either degenerating or regenerating cells.

FIG. 8.—Several large (8–14 μ) light-blue to colorless, basophiles, showing dispersed granules, vacuolization, pycnotic nuclei, and 6 μ vesicular nuclei.

FIG. 9.—Chromophobes with little cytoplasm, 3 μ vesicular nuclei, and indistinct cell membranes.

PLATE III

(Magnification: 150 × for Figs. 10–19; 250 × for Figs. 20–21)

FIG. 10.—Testis of a 9-day hypophysectomized snake, showing a narrow ring of germinal epithelium with sloughed cells in the lumen undergoing pycnosis and the small clumped interstitial cells. Control for Fig. 11. (No. 7F, 7-18-42.)

FIG. 11.—Testis of a 9-day hypophysectomized snake, maintained and stimulated by ten male pituitary homeo-implants. (No. 1F, 7-18-42.)

FIG. 12.—Testis of a 16-day hypophysectomized snake, showing shrunken seminiferous tubules consisting of a ring of spermatogonia and Sertoli cells, cellular debris in the lumen, and interstitial cells with irregular nuclei and vacuolated cytoplasm. Control for Fig. 13. (No. 8F, 7-25-42.)

FIG. 13.—Testis of a 16-day hypophysectomized snake which received a total of 350 I.U. of equine gonadotropin, showing marked stimulation of spermatogenesis and interstitial cells. (No. 11F, 7-25-42.)

FIG. 14.—Testis of a 66-day hypophysectomized snake, showing the completely disrupted germinal epithelium, the vacuolated, disintegrating cellular debris, and atrophy of the interstitial cells. (No. 23F, 9-4-42.)

FIG. 15.—Testis of normal September control, showing a second wave of spermiogenesis, the large interstitial cells, and the abundant intertubular colloid. Cf. Fig. 14. (No. 36F, 9-4-42.)

FIG. 16.—Normal April testis, showing spermatogonia and primary spermatocytes, the final disintegration of left-over cells in the lumen, and the large interstitial cells. (No. 15.)

FIG. 17.—Normal August testis, showing marked spermiogenesis, the dispersed small interstitial cells, and the abundant intertubular fluid. (No. 53.)

FIG. 18.—Normal October testis regressing in size, showing the unevacuated cells and vacuolated interstitial cells (*arrow*). (No. 83.)

FIG. 19.—Normal December testis from hibernating snake, showing the collapsed seminiferous tubules with spent germinal epithelium, the large vacuolated interstitial cells (*arrow*), and the small interstitial cells with an irregular nucleus. (No. 93.)

FIG. 20.—Pituitary of a castrated male, showing numerous small deep-blue basophiles (*B*) extending over a wider area than in the normal control, Fig. 21. Few pink acidophiles (*P*). (No. 27F, 9-4-42.)

FIG. 21.—Pituitary of a normal male, showing small deep-blue basophiles lining the sinusoids (*S*) and numerous chromophobes (*C*). (No. 36F, 9-4-42.)

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

THE CHEMISTRY AND PHYSIOLOGY OF HORMONES,[1] EDITED BY FOREST RAY MOULTON

Reviewed by ROBERT GAUNT
New York University

THIS volume grew out of a research conference of five days' duration held at Gibson Island, Maryland, in the summer of 1943, under the auspices of the American Association for the Advancement of Science. It is not intended to be a summary of all endocrinology, but it does constitute an excellent treatment of certain selected topics on the field. It is comparable to the volume published by the Biological Laboratory of Cold Spring Harbor as a result of its symposium on internal secretions in 1937; and the finished papers, apparently edited with care after the conference, probably represent the fruits of the conference discussions as a whole.

The phenomenal progress in the field of endocrinology is shown by comparison of this volume with that coming six years earlier from the Cold Spring Harbor symposium. In many of the fields, notably in that on the adrenal cortex, sound facts have been added in tremendous number and rational interpretations hence made possible.

The volume did not appear until eighteen months after the conference, and that it does not seem already out of date is due to two facts: first, the war has slowed the turbulent progress of endocrinology to such a pace that relatively little has been done in the last two years; second, publication was delayed long enough that the authors of the various chapters had time to include 1944 references in numerous instances.

The book is remarkably free of contradictions in a subject that has had many controversies, but a few minor ones are apparent. For instance, Friedgood (p. 202) states: "FSH causes the ovarian follicle to secrete estrogen"; but this is hardly consistent with the evidence presented by Fevold (p. 153).

White furnishes an excellent summary of our knowledge on the thyrotrophic, growth, and adrenotrophic hormones; and of particular value are his critical comparisons of assay methods and experimental data from different laboratories.

Marks and Evans give a more elaborate treatment of the physiology of the pituitary growth hormone, including its morphological and metabolic effects. They consider its relations, synergistic and antagonistic, to all the various other hormones concerned with growth and its possible identity with the "diabetogenic" and "myoglycostatic" factors of pituitary extracts, and they conclude that it can exert its characteristic action in the absence of any other hormone with the possible exception of insulin.

Jensen provides a review of the chem-

[1] Washington, D.C.: American Association for the Advancement of Science, 1944. Pp. 243. $4.00.

istry of insulin, concluding that its characteristic action depends on the protein molecule as a whole rather than on some prosthetic grouping. Lukens summarizes the physiological knowledge of the pancreatic hormone and discusses critically various theories concerning its action, but the keynote of his discussion lies in the frank recognition of the fact that "the exact mechanism of its action remains a mystery."

The papers by Kuizenga on the chemistry and by Ingle on the physiology of the adrenal cortex summarize a decade of progress that is highly satisfactory to those, such as the reviewer, whose interest in this gland dates from 1930 or before. At that time the only essential known facts were that this organ was necessary for life and that a crude extract made from it would serve as a substitution therapy. Kuizenga considers that there are two major remaining chemical problems. These are (*a*) to elucidate the nature of the highly potent "amorphous fraction" of cortical extracts and (*b*) to discover a method of practical synthesis of the 11-oxygenated steroids. Ingle's discussion makes clear that the remaining physiological problems, if not more difficult, are at least much more numerous.

In a brief introductory consideration of the adrenal medulla, Ingle appropriately emphasizes that a plethora of knowledge on the dramatic pharmacology of epinephrine is accompanied by a great paucity of data on deficiency phenomena in demedullated animals—data necessary to establish the physiological significance of many effects of injected epinephrine.

Irving gives a brief and conservative review of the physiology of the neurohypophysis, leading to the conclusion that an antidiuretic hormonal action has been demonstrated but that the physiological significance of the other pharmacological effects of posterior pituitary extracts is yet to be established. Most investigators would agree with this view; but the pertinent, though puzzling, experiments of Haterious and Ferguson on the hormonal nature of oxytocin were not considered. Irving points out that the contention of Abel that all activity of glandular extracts was in its natural state contained in one large mother-molecule has been vigorously resuscitated by Van Dyke and associates, who have probably isolated such a molecule in homogeneous form. It is still clearly true, however, that mild fractionation procedures yield two separable products of small molecular weight, namely, the pressor and the oxytocic principles.

The complex story of the pituitary gonadotrophic hormones, now generally agreed to be three in number, is well on its way to comprehensibility. This is indicated by the brief review of Chow on the chemistry and the more complete account by Fevold on the physiology of these substances. These summaries represent another great record of progress since the pioneer observations of Smith, Zondek, Evans, and others barely two decades ago.

Knowledge of the gonadotrophic substances of nonhypophyseal origin, principally chorionic and equine gonadotrophin, is in a less satisfactory state. Their chemistry is considered by Gurin, and their physiology by Levin. The latter author distinguishes between the biological effects of these various substances but concludes: "Although the gonad-stimulating substances of human and equine pregnancy are usually referred to as hormones, there is, in fact, no evidence to show that either substance satisfies the qualifications of this group of activators." More recent work, such as that of Hisaw and his colleagues on the function

of chorionic gonadotrophin, may make easier the re-writing in subsequent years of Levin's section on the *raison d'être* of these products of pregnancy.

No comprehensive treatment of the physiology of the sex hormones is included, but a concise review of the chemistry and synthesis of steroid hormones is given by Schwenk. It is in relatively nontechnical terms and can be understood by others than chemists. Gallagher reviews the status of the newer work on steroid metabolism, giving a good critique of the technical problems involved, such as those on the methods of hydrolysis of urinary steroids.

Friedgood duplicates this in part but emphasizes the problem of the 17-ketosteroids and their somewhat mysterious clinical significance.

Salter has written an excellent treatise on the metabolism of the thyroid hormone and the physiology within the thyroid gland itself. He shows that measurement of circulating thyroid hormone (organic I) gives a more correct and informative picture of glandular function than do morphological observations of the thyroid tissue.

Thompson's paper treats certain current problems concerning the antihormones, emphasizing possible clinical application of knowledge of these substances. He reports negative results in attempts to use antigonadotrophic materials as abortifacient agents in the human species.

Greengard reviews briefly the status of the large number of established and alleged hormones of the gastro-intestinal tract. The space accorded to each of these substances is unfortunately brief for this relatively ignored phase of endocrinology, but adequate bibliographic citations compensate for this lack.

The Bibliography of all papers, covering thirty-three pages of text, is gathered alphabetically at the end of the volume. No index is included, and the Table of Contents gives only chapter titles. If the latter had been expanded, specific material in which a reader is interested could have been more easily found. The discussion which accompanied the papers is not included.

In general, the volume is one of outstanding worth and serves to illustrate the value of symposia of this type.

THE EMBRYOLOGY OF BEHAVIOR,[1] BY ARNOLD GESELL, IN COLLABORATION WITH CATHERINE S. AMATRUDA

Reviewed by WILLIAM F. WINDLE
Northwestern University

PROFESSOR GESELL has contributed another book to a growing list of monographs from the Clinic of Child Development at Yale University. It is a cleverly organized and attractively printed volume, employing such devices of scholarly composition as Frontispiece, atlas section, appendixes, and list of selected references. The book will undoubtedly appeal to a large number of readers.

It is a little difficult to be certain for whom the book was written, whether the general public, physicians, psychologists, or biologists. In certain places it seems that the author must be writing for the public, perhaps for the benefit of mothers of prematurely born infants. As one reads along, however, one encounters phrases, sentences, and paragraphs

[1] New York: Harper & Bros., 1945. Pp. xxii+289+392 illus. $5.00.

couched in technical terms and philosophies so dependent upon detailed scientific knowledge that it appears quite impossible for the author to be directing his words toward the laity. On the other hand, many parts of the book are not strictly scientific, and those sections of it which should be often fail to attain this goal. Surely, the author is not writing as one scientist to another. His appeal to authority; his indirect way of stating relatively simple things which makes them seem profound; and his use of pleasant-sounding, although sometimes meaningless, combinations of words make it seem unlikely that he has a scientific audience in mind.

The monograph should be considered in two parts. The first nine chapters have to do with embryology of behavior and with fetal physiology. The rest of the book is concerned with prematurely born "fetal-infants" and "circumnatal infants." Factual material in the latter portion of the book is drawn from the author's own experience and illustrated with frames from motion-picture films taken in the course of his studies at Yale. The reviewer has had no experience with these phases of development in man and refrains from expressing opinions about these chapters. However, in the first nine chapters of the book misconceptions concerning fetal physiology will not be allowed to pass without criticism. A few examples will be cited.

The suggestion that the semicircular canals are supposed to be functional as early as the middle of the seventh week in the embryo and may result in righting reactions, which must be accomplished by changes in muscle tension and result in keeping the embryo on an even keel, lacks scientific evidence. Regrettably, such misstatements are sometimes perpetuated in the literature.

The author declares that well before the ninth week the fetus stirs; how he or anyone else has determined this is not stated. He does go on to say: "The eye of Man cannot, of course, witness the actual movements as they occur in the moist interior of the amniotic vesicle; but the mind's eye can visualize with approximation the manner in which the patterns of behavior become defined in their natural habitat." The line between fact and fancy is not clearly drawn.

The author holds it to be a fact that pre-respiratory movements serve to aspirate fluid into the air sacs and that this aspiration may serve a beneficial function by bringing about dilatation of the alveoli, facilitating the growth of their pavement epithelium. Here, as elsewhere, the author has accepted one investigator's experiments without reference to those of others which would have clarified the physiology of respiration in the fetus.

The author calls upon the authority of the late Dr. DeLee in support of the statement that some infants delivered by Caesarean section die because of retention of the *liquor amnii*, which is normally squeezed out of the lungs as the cylindrical thorax passes through the birth canal (*sic!*). The author also holds that the baby's very first breath may be an extensor startle which initiates an exhalation followed by inhalation. Apparently, his assumption that the lungs are expanded fluid in the normal fetus requires the first breath to be an exhalation.

On the subject of muscle tonus the author makes several statements which cannot be supported by fact. He holds that "in a primitive embryonic stage, tonus may be produced without nervous impulses through the sheer effects of the orientation of molecules." He also sup-

ports the theory of spontaneous firing of neurons—the electronic theory of nerve-cell activity. Some neurophysiologists will disagree with his discussion of electronic integration.

Probably the most severe criticism which can be made of Professor Gesell's book is failure to marshal scientific facts. Of course, this will not be apparent to the general reader, who gains only an impression of the subject anyway; but it would seem that a scientist does not need to sacrifice accuracy to popular appeal. When one becomes concerned only with the so-called broad principles and laws of nature and ceases to have patience to assemble and criticize individual items, facts, and details, he ceases to present his subject from the scientific point of view and allows it to fall into the classification of the metaphysical and the pseudoscientific.